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Final Report

Generating More Precise Post Mortem Interval Estimates With Entomological Evidence: Reliable Patterns of Gene Expression Throughout Calliphorid Larval and Pupal Development[1,2]

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1. Portions of this report were previously described in the doctoral dissertation of Aaron Tarone and the thesis of Kimberley Jennings (in preparation), in the Journal of Medical Entomology, and in the Journal of Forensic Sciences (submitted).

2. This project was carried out at Michigan State University. The study involved the joint effort of the following individuals: Dr. David Foran, PI, Aaron Tarone (Department of Zoology Graduate Program, Michigan State University), Kimberley Jennings, Erin Lenz (Forensic Science Graduate Program, Michigan State University), and Trevor McLean (Undergraduate, Michigan State University).

Abstract

Entomological evidence is widely used to estimate a postmortem interval (PMI) during death investigations. Blow flies (Diptera: Calliphoridae) typically colonize remains within hours of death. They lay eggs on carrion, which hatch and undergo a number of predictable developmental changes. Owing to the quick colonization and reliable progression of development, investigators can use historical temperature data, stage of development, established development tables, and larval body size to backtrack from the time of collection of blow fly evidence to the time of colonization—providing a minimum PMI estimate.

This straightforward process is complicated by a number of factors. During development the amount of time spent in each stage gets progressively larger. The pupal stage alone comprises approximately half of immature development, and at low temperatures can last well over a week. An extended development time means that PMI estimations made with flies at more advanced developmental stages must be given far larger error estimates, decreasing the usefulness of the data. Body size can be helpful in refining age estimates within a developmental stage, however it comes with the caveat that postfeeding larvae begin to shrink, while exhibiting much larger variance in body size than feeding stages, and that pupae do not change in size at all. Therefore, the use of body size can only help refine the age estimates for feeding larvae, which represent just the first quarter of immature development.

In the research described here, gene expression information was incorporated into the age estimation process in order to better define a PMI. Genes exhibit myriad expression profiles, and by adding data from an informative suite of genes with different profiles, it should be possible to more precisely age blow flies at all developmental stages. The expression levels of informative 9 genes were assessed in 958 immature *Lucilia sericata* (a globally distributed and forensically

useful blow fly) larvae and pupae, using quantitative PCR. Generalized additive models (GAMs) were used to predict immature development percents, incorporating developmental stage, body size, and gene expression information, significantly increasing the precision and accuracy of blow fly age predictions.

The method of predicting blow fly age was then validated in a blind study. Models incorporating body size and developmental stage with and without gene expression were used to predict the ages of 90 flies. Models that contained gene expression profiles were notably better at predicting fly age. This was particularly true for post-feeding third instar larvae and pupae, which are the most difficult developmental stages to age using standard procedures.

Additional projects were required to accomplish the major goals of this research. Methods for high throughput quantitative analysis of gene expression data were perfected. A standard operating procedure was developed for rearing *L. sericata* that more precisely mimicked how flies grow on carrion. Profiles of 55 larvae that failed to pupate were produced—individuals that would be misleading to an entomologist attempting to estimate a PMI. Through gene expression data, such flies were identifiable. Finally, the influences these new methods have on the field of forensic entomology, as well as how they help meet the scientific requirements of Daubert, were considered.

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Executive Summary

Introduction

Blow fly evidence can be useful in estimating a post mortem interval (PMI) during death investigations. This utility is largely due to the reliable development of blow flies and their predilection for colonizing remains within hours of death. Owing to this, investigators can utilize blow fly evidence as a biological clock, using the age of evidentiary flies to backtrack to the time that remains were colonized. Such a period is a good indicator of the minimum PMI, as death is very rarely preceded by blow fly colonization.

There are a number of factors that decrease the precision of entomologically based PMI estimates however, which are largely consequences related to the specifics of blow fly development. Flies generally lay hundreds of eggs on a corpse within hours of death. The eggs hatch into larvae, which feed and grow on the remains. As larvae increase in size, they must molt their cuticle. Larvae molt twice, separating the larval stage into three segments (instars). The first two instars are devoted to feeding and growth. During the third instar, larvae feed for a time, then hormonal signals initiate the cessation of feeding and the beginning of metamorphosis into an adult fly. After a few days the larvae form a puparium and metamorphose. Eventually, the pupae eclose as adult blow flies.

The progression of development transpires in such a way that each stage is successively longer than the next. As an example, pupation comprises (approximately) the last half of immature development, which depending on temperature can last well over a week. This means that as blow flies age, the resulting PMI estimates will come with progressively larger error rates, potentially encompassing a window of time greater than a week. Obviously this does not make for highly accurate PMI estimates.

One set of information that can be useful in refining age estimates within a developmental stage is body size. As larvae feed they increase in size in a relatively linear fashion. This enables the use of linear regression to more specifically age the feeding stages. However, this approach does not work for the postfeeding stage, as the cessation of feeding leads to a decrease in body size, with a much greater variance in size than during the feeding stages. Additionally, the pupal stage does not change size, so it cannot be used to refine pupal age estimates. Unfortunately, the feeding stages comprise only the first ~25% of immature development. To make more precise age estimates of blow fly age in the latter developmental stages, it will be necessary to incorporate new information into the PMI prediction process, ideally independent of developmental stage and size. Such data should provide information that is descriptive of development in all stages, especially within postfeeding third instars and pupae.

Fitting this criterion is gene expression data. During development, a variety of genes must be up- and down-regulated. Indeed, a great deal is known about the regulation of gene expression throughout fly development and many genes are regulated during this process. Specifically, research in *Drosophila melanogaster* (a fly species and the closest model organism to blow flies) shows that a tremendous level of gene expression change exists throughout fly development, meaning a detailed description of development is possible through profiling a handful of genes, if the right suite of genes is chosen. Importantly, the procedures necessary to analyze gene expression are very similar to those already done in a typical crime lab that is capable of analyzing DNA. As such, any gene expression protocol should be easily implemented in any DNA crime lab in the US.

The research undertaken here utilized gene expression information in age estimates of the green bottle fly, *Lucilia sericata* (Diptera: Calliphoridae) (Meigen). The species was chosen

because it is forensically useful and globally distributed. Additionally, there were already a number of gene sequences available for *L. sericata* and a sister species (*Lucilia cuprina*). This limited the number of new gene sequences that needed to be produced, providing sequence data for a workable set of loci with minimal effort.

Before genetic aging of blow fly cohorts could be undertaken, experiments were necessary to develop a laboratory rearing protocol that would provide a developmental progression most appropriate to rearing conditions on carrion. The experiments were a consequence of divergent rearing protocols found in the four publications detailing *L. sericata* development times, and a lack of connection between laboratory growth and growth under realistic conditions (on carrion). In addition, each of the four studies was conducted with different strains of *L. sericata.* Quantitative genetic theory dictates that factors like minimum development time are continuous traits, and are determined by both genetic and environmental factors. Since both of these differed among the earlier studies, it was impossible to determine the cause of variation among published fly growth data sets. Consequently, thirty-seven cohorts of *L. sericata* from the same genetic strain were raised under various environmental conditions. Once optimal growth rates were determined, laboratory growth was compared to development of *L. sericata* on rat carcasses. The laboratory growth that was most similar to the growth of this species on rats was used in the sampling protocol for the gene expression work.

Next, six cohorts of *L. sericata* (two each from California, Michigan, and West Virginia) were raised and sampled, yielding length, weight, and developmental stage data. Time series growth curves were obtained for each cohort at 20ºC and 33.5ºC, based on twice-daily collections of larvae and daily collections of pupae. The collections yielded basic stage and body size information and provided the individuals needed for gene expression analysis.

Statistical modeling of the six cohorts was conducted using generalized additive models (GAMs). This was important for two reasons: first, growth is non-linear, and the ability to model non-linear curves might improve age predictions of postfeeding third instars. Using non-linear statistics had the potential to account for the decrease in body size during the third instar, thus reducing the inaccuracies associated with predictions made with this problematic group. Second, gene expression also follows a non-linear pattern, thus an appropriate method was required to make predictions with genetic data. GAMs are likelihood statistics that are capable of incorporating multiple linear (e.g. developmental stage and genetic strain) and non-linear data (e.g. length, weight, and gene expression curves) into one statistical model, which can be useful in predicting a variable of interest (e.g., age). In addition, it was possible to determine the relative effects, statistical significance, and error rates generated using each variable (length, weight, stage, strain, temperature, and subsequently gene expression).

An initial study of age estimation through gene expression was undertaken using three genes. This was done on fly eggs, as this is a very brief portion of development, and eggs cannot be aged by other means (e.g., size does not change). Next, cDNA from a large subset of the samples measured for the GAM study were analyzed for the expression of 12 genes throughout immature development. After ~100 samples had been analyzed, any genes determined to be non-informative were removed from those profiled. Ultimately, expression levels for 9 genes were used to construct and assess models predicting the age/development of the species.

To validate predictions made with GAMs, a blind study of fly age estimation was conducted. Ninety individuals were sampled from cohorts raised on rats at 20ºC, 33.5ºC, and at ambient temperatures by an independent researcher. The flies were weighed, measured, and staged, then assessed for the expression levels of the informative genes. The quantitative PCR

(qPCR) profiles were entered into GAMs created from the database, and their predicted development percents were compared to their true development percents, validating the use of gene expression for PMI estimates with blow fly data.

During the rearing of flies, larvae were regularly observed that did not progress to eclosing adults, which if collected from a body could lead to underestimation of a PMI. The arrested development of these "Peter Pan" individuals could be explained by a number of phenomena including mutations of genes critical to development, larval diapause (a slowing of insect development akin to hibernation), estivation (stress induced developmental delays), or just naturally slow development. Since the gene expression profiles of such individuals might be useful in distinguishing them from normally developing third instars, they were sampled on the day that adults from their cohort eclosed. Fifty-five individuals were profiled for the expression of the 9 informative genes, yielding four graphical differences in gene expression when compared to normally developing postfeeding third instars, three of which were statistically significant. Likewise, a number of individuals were sampled in the blind study. Their profiles were compared to the profiles of known "Peter Pan" flies demonstrating that such flies can be distinguished from normally developed flies by the expression levels of a few genes. The ability to detect "Peter Pan" larvae is useful as it can be an indicator that pupae existed but were not sampled at the crime scene, and will enable investigators to avoid PMI predictions based on developmentally irregular individuals.

Blow Fly Collection and Rearing Methods

L. sericata strains were obtained from the Michigan State University campus in East Lansing, MI, the UC Davis campus in Davis, CA and the West Virginia University campus in

Morgantown, WV. Species was determined visually and through sequencing of the cytochrome oxidase 1 mitochondrial gene

Individual cages were maintained for each strain, with multiple generations kept in a cage, and multiple females contributing to the next generation. Cages were continually supplied with honey and water. To induce oviposition, cages were introduced to ~1mL of beef blood. The next day, a slice of liver was placed in cages and observed until females were seen laying eggs. For timed experiments females were allowed to lay eggs for 1hr. If strains were simply being maintained, females were allowed to lay until ~1000 eggs were laid. The eggs and liver were placed in 1L canning jars and a breathable cloth lid. For pupation, larvae were provided 500 mL of vermiculite for strain maintenance or 500 mL sand for experimental research.

The freshness of liver, moisture of liver, destructive sampling, and the freshness, location, and type of pupation substrate were studied to determine their effects on development time. 2559 individuals were examined. Freshness of liver was tested by providing ~40g of liver daily or ~120g of liver every third day to a cohort of flies. Moisture of liver was examined through the presence or absence of a moist paper towel in the jars. Destructive sampling involved removing 12 individuals daily from the cohort. Pupation substrate was tested by providing cohorts with sand or vermiculite when the postfeeding third instar stage was attained, and substrate freshness by transferring 125 individuals from the jars with liver to new jars with ~500 mL of fresh sand or vermiculite. Thirty-seven cohorts were raised in different combinations of the treatment types, and the effects were assessed statistically for their effects on the length and advancement of developmental stages.

The growth of the laboratory reared flies was compared to the growth of flies on rats. Sprague-Dawley rats were obtained from the Michigan State University Laboratory Animal

Resources (MSULAR) under the ethical guidelines of that organization. Rats were sacrificed by CO_2 asphyxiation and kept in a sealed plastic bag for 0–2 days. On the day rats were obtained fly cages were presented with liver. Egg masses were collected normally and transferred to the mouth of the rat.

Once a laboratory growth rate that approximated development on rats was ascertained, the collection of flies for gene expression work was undertaken. Cohorts of eggs were split into two treatments raised at 20ºC and 33.5ºC, at a 12:12 h light cycle, and 25±5% relative humidity. Ten larvae were collected twice daily, once in the morning and once at night. Developmental stage was determined, and flies were measured to the nearest ½ mm by observing their maximum extension, and weighed to the nearest $1/100^{th}$ of a mg on a microbalance. Pupae were sampled based on the day that they formed a puparium (0–1 days old, etc.). Five individuals per time point were used in the production of a gene expression database.

Molecular Methods

Gene sequences were obtained from www.ncbi.nlm.nih.gov or by using the sequence of related species to design primers for the appropriate loci. Genes available on line included *ribosomal protein 49* (*rp49*), *resistance to organophosphate 1* (*rop-1*), *heat shock protein 60* (*hsp60*), *heat shock protein 90* (*hsp90*), *wingless* (*wg*), and *slalom* (*sll*).
Genes sequenced in-house were ß *tubulin 56 D*, *chitin synthase* (*cs*), *acetylcholine esterase* (*ace*), *ecdysone receptor* (*ecr*), *ultraspiracle* (*usp*), *scalloped wings* (*scl*), *white* (*w*), *rhodopsin 3* and *cytochrome oxidase 1 (CO1)*. Primers for qPCR were then designed and optimized.

Given the large quantity of RNA, cDNA, and qPCR samples that were involved in this project, a number of high throughput methods were adopted. RNA was collected in a 96-well

format using an ABI PRISM Nucleic Acid PrepStation 6100. DNased RNA samples were used to make cDNA, and quantities determined using an ABI 7900HT real time thermocycler and SYBR Green technology.

Statistical Methods

Statistics were analyzed using the free and publicly available R statistical program (R Development Core Team. 2004). A number of statistics were generated. During the fly rearing research a type III ANOVA was used to assess the effects of treatment types on development times. Models were constructed using all variables for each developmental stage and only variables significant at the $a<0.05$ level were considered to have an effect on the duration of a specific developmental stage. Total development time was assessed with a model testing all variables that were significant at any developmental stage (i.e., Total development = Meat Freshness + Paper Towel + Transfers + Destructive Sampling).

Non-linear curves were constructed for length, weight, and gene expression levels throughout development, using lowess curves, with smoothing parameters appropriate for the data. Non-linear confidence intervals were also constructed. Development percents were calculated by dividing the time of collection minus the time of the first observed eggs by the minimum development time. GAMs were used, to predict the immature development percents of individuals.

Results

Sequencing results were successful for *COI* and the nuclear genes. Flies were identified as *L. sericata* through *COI* sequence, with all sequences receiving their closest identity matches

to that species in BLAST searches at www.ncbi.nlm.nih.gov. In addition, *L. sericata* sequences for the genes ß *tubulin 56D*, *cs*, *ace*, *ecr*, *usp*, *w*, *scl*, and *rh3* were obtained from samples that yielded *L. sericata COI* sequence.

At least one pair of qPCR primers for each of the genes yielded amplification of a single PCR product of the size expected. Single products were confirmed by gel electrophoresis and dissociation curves from qPCR reactions. Once successful primer pairs were identified, concentrations of forward and reverse primers were optimized.

During fly rearing *L. sericata* development was observed to be plastic (variable), at a level that alone could explain all published differences among fly development data. The provisioning of fresh liver each day significantly shortened the duration of the feeding portion of the third instar. The presence of moist paper towels further shortened the duration of this stage. Transferring postfeeding third instars to ~500mL of fresh sand or vermiculite significantly shortened the amount of time spent in that stage. Likewise, destructive sampling resulted in a significantly longer pupal development.

To determine which laboratory growth rate best mimicked that on a body, development was compared to flies raised on rat carrion. Rat reared cohorts grew as fast as the fastest growing liver-raised cohorts. A comparison of growth curves for rat and liver fed flies demonstrated the greatest similarity to treatments that were given fresh liver daily on moist paper towels.

Using data from the 2559 individuals sampled, a comparison of 18 GAMs, utilizing combinations of developmental stage, length, weight, temperature, and strain pinpointed key factors that proved to be important in predicting blow fly age. The first was that developmental stage is the single most informative piece of information for predicting development that is

currently used by forensic entomologists. Second, all tested variables were significant predictors of development in at least one model, though some exerted more influence on predictions than others, with length and weight explaining less of the deviance in development that temperature and strain. Third, length and weight were only useful for refining age predictions of feeding larvae. Finally, when the models were used to predict the development of the rat-raised cohorts, their performance was very similar to the predicted performance. This demonstrated that even if a model is lacking information, its predicted performance is a good indicator of its ability to estimate age, allowing investigators the flexibility to work with the data they receive.

The results from the egg gene expression study showed that using just 3 genes it was possible to break egg development into distinct stages with unique gene expression profiles. From 0–2 h of age *cs* was never expressed and *sll* and *bcd* were expressed at their highest levels. From 2–9 h eggs expressed *cs* at increasing levels and *bcd* and *sll* were expressed at relatively low levels. A GAM utilizing the expression levels of all genes (after 2 h) predicted egg masses within 2 h of their true age for 91% of the egg masses used to develop the model. The experiment confirmed the underlying theory of the research: that gene expression is predictably variable throughout development.

Following this pilot study, the gene expression profiles of larval and pupal stages were thoroughly investigated in 6 replicates of flies (two each from the CA, MI, and WV strains) raised at 33.5ºC and in 4 replicates of flies (two each from the CA and MI strains) raised at 20ºC. After ~100 samples were collected it was determined that *wg*, *scl*, and *rh3* would not provide useful information due to either large variance in expression or expression changes that provided the same information as developmental stage. Accordingly, the next 958 samples were only

profiled for the expression levels of the housekeeping genes and *cs*, *hsp60*, *hsp90*, *ace*, *ecr*, *rop-1*, *w*, *usp*, and *sll*.

From the resultant database, 23 GAMs were created and assessed for their predicted ability to estimate development percent using some combination of the variables measured (stage, length, weight, temperature, strain, gene expression). All genes were significant predictors of development percent either by themselves or in models that included some combination of development stage, strain, and temperature. Also, the binary (e.g., expressed or not expressed) expression levels of four genes (*cs*, *ace*, *w*, and *sll*) were statistically significant predictors of development percent.

In comparing possible gene expression-based GAMs to other GAMs from the same database, several points became apparent. First and foremost, incorporating gene expression data drastically improves the ability of a model to predict blow fly age. The most drastic improvements were in enabling far more precise predictions of pupal and postfeeding third instar development. Additionally, the use of genetic variables in a GAM resulted in more evenly distributed error as predictions of pupal and postfeeding larval development percent approached the precision of predictions made for feeding larvae. Percent deviance explained (PDE) and generalized cross validation (GCV) scores also improved for models that included gene expression data, again, most notably in the latter stages. The PDE for all of development increased from 88.2% for a GAM predicting age with stage alone to 94.6% for a model that included all variables, but for the difficult to age third instar and pupae, these values went from 36.2% to 79.8%, and 15.8% to 78.2% respectively. Overall, the results indicated that predictions with the models should be far superior to standard forensic entomological techniques.

To test whether the models performed as predicted, a blind aging study was conducted. An independent investigator collected samples from cohorts of Michigan *L. sericata* raised on rat carrion. Three rat raised cohorts were used, one at ambient temperatures, one at 20ºC, and one at 33.5ºC. RNA was collected from 90 sampled flies and 75 provided full or partial profiles, which were used to predict development percent with the appropriate GAMs. The predicted ages were compared to the true ages recorded by the independent researcher. Once again, models that used gene expression were superior in predicting development percent and were capable of predicting age within ~10% of true development percent at all developmental stages.

Gene expression profiles were also useful for identifying developmentally retarded postfeeding third instar larvae, i.e., individuals who failed to mature into adults, that could easily lead to an artificially short PMI estimate. The gene expression profiles of 55 "Peter Pan" flies were plotted against gene expression in normally developed flies. Four genes (*hsp90, rop-1*, *usp,* and *sll*) were identified as having graphically different expression levels in postfeeding third instars when their expression was compared to "Peter Pan" flies (as defined by the middle 50% of expression data for a gene in non-pupating flies being past the median expression level for that gene in postfeeding third instars). Of these, all but *usp* were differed significantly as measured by F-tests. Several such flies were sampled in the blind study and also produced incongruent age predictions. In these samples, three of the same loci demonstrated expression level similar to those of known "Peter Pans", indicating that thee loci are worthwhile markers for the "Peter Pan" condition and are useful in identifying postfeeding third instars that should not be used to predict blow fly age. Clearly, the ability to detect such individuals is important for making more precise PMI predictions with entomological evidence as it enables investigators to avoid erroneous age predictions.

Conclusions

The research undertaken has advanced the ability of an investigator to estimate a PMI derived from blow fly evidence by providing new tools, tools that significantly increase the accuracy and precision of age predictions, particularly at the most difficult to age developmental stages. The basic theory upon which the entire project was based, that gene expression can provide more precise age estimates, was established, and was most successful in developmental stages that are currently the most difficult to assess. At the same time, laboratory rearing conditions for blow flies that most accurately mimic those on a cadaver were generated, creating a standardized operating procedure that helps meet the tenets of Daubert. Likewise, the modeling of larval growth (length and weight), along with gene expression, allows for confidence intervals and error estimates to be produced, also required under Daubert. In total, the works performed have produced a substantial leap forward in using entomological data to more accurately estimate time since death.

Introduction and Background

Forensic entomology is a powerful tool that can aid in estimating a minimum postmortem interval (PMI) during death investigations (Catts and Haskell 1990). This application is possible due to the tendency of blow flies (Diptera: Calliphoridae), and other less common necrophagous flies, to colonize remains within hours (or less) of death, and thereafter proceed through a series of developmental stages. Female blow flies lay eggs around orifices or wounds that hatch into larvae, which feed on a body and grow through three larval instars. Each instar is separated by a molt of the cuticle that enables further larval growth. During the third instar, larvae cease feeding and (usually) leave the body to form a puparium. Within the puparium, the fly experiences metamorphosis and eventually ecloses as an adult fly.

This developmental process is predictable, and has long enabled investigators to use larval development tables of forensically useful species (e.g. Kamal 1958) to predict the ages of immature flies associated with remains. Insect development is also dependent on temperature (Anderson 2000; Grassberger and Reiter 2001), so if investigators know the developmental stage of the oldest flies collected as evidence and has historical weather data for the scene, they can determine the window of time necessary for a species to develop to that stage. That period of time is generally assumed to be the minimum PMI.

This basic procedure has been the accepted technique for predicting a PMI from insect evidence for decades. The static nature of the approach is due, in part, to the general success of the method, which has been upheld numerous times in American and international courts (Greenberg and Kunich 2002). However, this lack of advancement has resulted in the persistence of a number of caveats associated with PMI predictions that are based on fly evidence. One problem is that, since each developmental stage gets progressively longer through

fly development, a PMI prediction obtained from later stages will necessarily include a much larger window of time. For example, Kamal (1958) found that, for the species *Lucilia* (aka *Phaenecia*) *sericata*, growing at 26.7°C, the second larval instar lasted 9–26 h while the pupal stage lasted 5–11 days. At lower temperatures the pupal stage of this species can last even longer (Anderson 2000; Grassberger and Reiter 2001), and age/PMI estimates must be correspondingly broad.

One method for generating a more precise age estimate within developmental stages is to include body size in the PMI prediction process. As blow fly larvae feed, they increase in size in a generally linear fashion, with relatively little variance (Wells and Kurahashi 1994, Grassberger and Reiter 2001; Greenberg and Kunich 2002). At this point in development linear regression can be used to refine age estimates. However, the approach highlights the second caveat: larvae shrink when feeding ceases during the third instar (Wells and Kurahashi 1994, Grassberger and Reiter 2001; Greenberg and Kunich 2002) and exhibit a larger variance in body size than previous stages. Additionally, pupae do not change in size as they age. These facts mean that the last two (and longest) developmental stages provide imprecise (though accurate) PMI estimates, due to the uncertainty stemming from their long durations, variance in body size, or unchanging body size.

The last caveat associated with the status quo approach for predicting blow fly age is that it can sometimes be difficult to distinguish between feeding and postfeeding third instar larvae. The distinction between the two portions of the instar depends on qualitative data related to the visibility of tissue in the crop (indicating a feeding third instar) and the behavioral change in feeding (Anderson 2000). If investigators do not note the behavior of larvae when they collect evidence, the only physical evidence a forensic entomologist will have to work with is the

visibility of tissue in the crop. Several factors, including starvation of larvae and the dilution of crop contents into storage solution over time, compounded by the similarity in sizes of feeding and postfeeding third instars, can make distinguishing between the feeding and postfeeding stages of the third instar difficult or impossible (Anderson 2000).

In these cases it is clear that the shortcomings of the current forensic entomological approach will not be addressed until new types of data are included in the PMI prediction process. The main focus of the research detailed below was to improve the precision of blow fly based PMI predictions through the use of gene expression information. With the arrival of the genomic age biologists have developed new tools that have enabled them to assay gene expression levels at relatively low cost. From these tools a detailed understanding of gene regulation has emerged (reviewed in Kalthoff 2001). As eukaryotes (including blow flies) develop, a variety of proteins are required, thus the cellular transcriptional machinery initiates the expression of more RNA from those genes. Given the highly specific control development is under, the expression levels of developmentally regulated genes can be predictable as they are up- or down-regulated. Predictable gene expression patterns have the potential to aid forensic entomologists in more accurately estimating blow fly age.

In addition to basic gene expression theory, much is known about the expression of genes throughout the development of fly species. One of the major model organisms in modern biology research is the fruit fly *Drosophila melanogaster*. This species, like blow flies, belongs to the group of flies known as the "higher flies" or Cyclorrhapha. The development of all Cyclorrhaphan flies is very similar, including three larval instars and the formation of a puparium (McAlpine 1989). This means that genes known to vary throughout the development of *D. melanogaster* are excellent *a priori* candidates for study in the context of forensic

entomology, as the similarities among these flies indicates that blow fly genes are likely to be expressed in a similar manner to (Ali et al. 2005, Mellenthin et al. 2006).

Two recent genomic studies in *Drosophila* (Arbeitman et al. 2002 and Beckstead et al. 2006) have demonstrated that thousands of genes have predictable and temporally variable gene expression. Of these, there are myriad expression patterns and each can be used to indicate different points in development. For example, in *D. melanogaster*, the gene *Amalgam* is expressed at its highest levels during early pupation, while CG17814 is expressed at its highest levels during late pupation (Arbeitman et al. 2002). In this case, knowing the expression levels of both of these genes could help distinguish between early, middle, and late pupal development. Such knowledge led to the hypothesis tested herein—that by analyzing the right combination of developmentally regulated genes, it will be possible to identify more specific points in fly development than by current forensic entomology techniques.

Though knowledge of gene expression regulation in *D. melanogaster* demonstrates a theoretical capacity to predict blow fly age, gene expression profiles must be produced in a forensically useful blow fly species. The species studied in this research was *Lucilia sericata,* because it is a common fly encountered in forensic entomology and it is globally distributed. The *Lucilia* genus has also been included in multiple molecular studies, mostly due to the economic effect of *L. cuprina* infestations of Australian sheep. This means that gene sequence information can often be obtained from the public domain, or easily sequenced in *L. sericata* by designing polymerase chain reaction primers from *L. cuprina* sequence, thereby limiting the effort spent on acquiring gene sequences.

The report below is divided into multiple sections, which detail experiments addressing several questions. Because each set of experiments dealt with different problems and used

different methodologies, they are presented as separate units, although naturally, some portions occurred concurrently. The first set of experiments was related to rearing flies. Four earlier publications contain laboratory data on the growth of *L. sericata* (Kamal 1958; Greenberg 1991; Anderson 2000; Grassberger and Reiter 2001), each of which outlines different growth rates for the species. None of the authors, however, compared growth under laboratory conditions to growth on carrion. Hence a series of experiments was undertaken to determine how the different rearing methods described in the literature compared to one another, and which one best mimicked growth on cadavers.

The second set of experiments dealt with methods for assessing the data obtained, both for fly growth and gene expression. Statistical tools were developed to make predictions with both the non-linear length and weight data, as well as gene expression profiles. Since genes are not typically expressed in a linear fashion throughout development and information from multiple genes would be necessary to more precisely predict blow fly age, a new type of statistical approach was required. A candidate method of analysis was first tested for its ability to predict age using length and weight data from the 2559 immature flies. Various generalized additive models (GAMs) were constructed and compared as to their abilities to predict blow fly age. The models use likelihood statistics to incorporate multiple non-linear variables into a prediction (Hastie and Tibshirani 1990, Wood 2006). Results indicated that accurate, though imprecise at later stages, predictions of blow fly age could be made using length and weight. The effects of temperature and strain were also considered, and both were significant variables that affected development, but their influence on predictions was small. The performance of one model was then assayed by using it to predict the age of flies in an independently derived data set

(flies reared on rats). GAMs also allow generation of error rates/confidence intervals, thus proved useful in meeting these considerations of Daubert.

The third set of experiments dealt directly with age and gene expression. The ability to determine fly age based on gene expression profiles was first tested in eggs, using a modest set of three genes. Eggs represent the shortest developmental stage in flies (~10 h), yet this stage was successfully subdivided into three separate sectors, showing the utility of the methodology.

From there, the forth and most extensive line of research was conducted. This involved the staging, measuring, weighing, and fixation of 2559 individual larvae and pupae. Of these, 958 were profiled for the expression of 9 developmentally regulated genes (three genes were removed as they were uninformative). Once all gene expression profiles had been obtained and the means of predicting age had been established, GAMs were constructed using gene expression levels, and compared to standard methods as to their abilities to predict blow fly age. Models that included gene expression data markedly increased the precision of age predictions. This was particularly true for third instars and pupae, which are the most difficult to age using standard techniques.

Following gene expression characterization of the loci using the flies of known ages, a blind study was undertaken in which larvae and pupae were reared on rats. The successful validation of the methodology was a critical part of the research, because the results of any statistical modeling must be confirmed on independent data. The blind study confirmed that the results are not specific to the data set used to make the models; gene expression data significantly improved the aging of flies from the blind study.

During and after the collections for the main quantitative PCR project, it was noted that some individuals in all replicates failed to form a puparium, even as adults were eclosing from

that replicate. Forensically, the collection of such individuals at a crime scene could be very misleading, drastically underestimating a PMI. However, it seemed plausible that the genetic profiles of these individuals might be informative, even with their misleading age appearance. Ten (or as many as were available) of the non-pupator or "Peter Pan" individuals were collected from each replicate. Of those ~120 individuals, 55 were profiled, and it was determined that they had predictable expression pattern differences from normal postfeeding third instars, which should help to identify misleading flies.

Plasticity in Fly Growth (The following section was published in the Journal of Medical Entomology 43(5):1023–1033 (2006) under Tarone and Foran)

Forensic entomologists rely on published data of blow fly development to estimate the time since initial colonization of remains, thus extrapolating a postmortem interval (PMI) (Catts and Haskell 1990). PMI estimates based on entomological evidence have been widely and successfully presented in legal proceedings, however the laboratory study of blow fly development, on which these estimates are founded, has never been standardized. Because of this, entomologists may utilize different blow fly developmental data sets, which can lead to variable PMI predictions. Further, a lack of scientific standardization has the potential to call into question the overall accuracy of entomological evidence (see Saks and Koehler 2005).

Prominent examples of differing laboratory rearing methods and resultant data sets can be found for the widely distributed green blow fly, *L. sericata* (Diptera: Calliphoridae) (Meigen) (Kamal 1958, Greenberg 1991, Anderson 2000, Grassberger and Reiter 2001). These data sets all present a developmental time scale from egg to adult. In his work, Kamal (1958) recorded only the duration of each developmental stage, while Grassberger and Reiter (2001) and Greenberg (1991) also measured the length of maggots until pupation, and Anderson (2000) measured crop length throughout development. Each of these studies utilized different fly-rearing techniques, varying in the quality and type of food, the quality of pupation substrate, and the destructiveness of sampling. Likewise, the authors measured fly development at different temperatures, and reported development data in assorted ways (minimum, average minimum, mode, and maximum growth). The resulting picture of *L. sericata* development is clouded, with relatively small differences in minimum development time among all studies, while Anderson

(2000) characterized a notably longer minimum development time at temperatures similar to the others. Unfortunately, direct comparison of these studies is impossible, as experimental conditions and genetic background of the flies varied among them. Further, even though the data sets were generated with a goal of relating larval development to PMI estimates on corpses, no attempt was made to tie laboratory-established growth rate data to ecologically relevant larval development on carrion.

Development time is a quantitative trait that is expected to vary due to both genetic and environmental factors (Mackay 2001; Conner and Hartl 2004). Understanding genetic and environmental effects on quantitative traits is best accomplished by altering one variable while keeping all others constant, and a limited number of such experiments have been conducted in a forensic entomological context. For instance, Kaneshrajah and Turner (2004) demonstrated that *Calliphora vicina* (Diptera: Calliphoridae) reared under otherwise constant conditions showed variable growth when raised on different organs, and Wells and Kurahashi (1994) indicated that differences in rearing protocols were the likely source of discrepancies regarding development times of *Chrysomya megacephala* (Diptera: Calliphoridae). Likewise, high-density rearing conditions that increase maggot mass temperatures were shown to shorten development times of *C. megacephala* (Goodbrod and Goff 1990). Recently, *L. sericata* was found to exhibit variable growth patterns depending on the species and tissue type on which cohorts were raised (Clark et al. 2006). Certainly it appears that rearing conditions can have a major impact on the developmental timing of calliphorids.

Just as environmental factors influence calliphorid development, intra-specific differences have the potential to produce variation in fly developmental times. The field of ecological genetics is replete with cases demonstrating the effects of genetic background on

quantitative traits (reviewed by Mackay 2001; Conner and Hartl 2004). Developmental variability has been documented in many fly species, including strains of *Drosophila* (Diptera: Drosophilidae) (Johnson and Schaffer 1973, Oudman et al. 1991, Hoffmann and Harshman 1999, Parsch et al. 2000), *Rhagoletis pomonella* (Diptera: Tephritidae) (Feder et al. 2003), and *Scathophaga stercoraria* (Diptera: Scathophagidae) (Blanckenhorn 2002). Since each *L. sericata* study referenced above was conducted on different populations, it is impossible to separate the effects of environment and genetics on fly development. Potentially, any (perhaps all) differences among *L. sericata* studies could be explained by genetic variation among strains, however this would only be demonstrated if each strain was raised using the same experimental protocol. Unless standard rearing conditions are adopted, such comparisons are impossible.

The potential influence of the environment and genetics on quantitative traits, and in particular development time, led to the hypotheses tested herein that *L. sericata* growth is plastic with respect to rearing conditions, and that fly development on carrion will best be predicted by a specific combination of laboratory conditions that affect this plasticity. Temperature and humidity are already known to affect development time (Greenberg 1991, Anderson 2000, Grassberger and Reiter 2001) and mortality (Wall et al. 2001) in this species, so these conditions were held constant to investigate the effects of other rearing variables. Likewise, the flies in these experiments originated from the same source population, allowing genetic differences to be largely ruled out as a source of developmental variation. By changing the exposure of a single strain of *L. sericata* to specific environmental conditions, several questions related to the hypotheses were addressed. In particular: 1) Do laboratory rearing conditions affect the development time of *L. sericata*? 2) Are any developmental differences caused by laboratory rearing conditions large enough to explain the variation observed among published growth data

on this species? And 3) Does growth generated under laboratory conditions accurately reflect larval development of *L. sericata* on a carcass?

Materials and Methods

Fly Collection and Rearing. *L. sericata* adults were collected from the Michigan State University campus in East Lansing, Michigan throughout the spring, summer, and fall of 2004, and were used to establish a general population cage of approximately 200 flies. Species identification was done using multiple keys, two independent identifications, and by comparing the DNA sequence of a 798 base pair region of the mitochondrial *cytochrome oxidase 1* gene to published sequences on the NCBI website using the BLAST link. Forward primers for DNA amplification were GATCAGTAGTAATTACAGCT, and TAATATTGCTCATGGAGGAG, while reverse primers were TTGACTTTTTAATATCTTAG, and CCTAAGAAATGTTGAGGGAAG. Polymerase chain reactions were run for 35 cycles by denaturing at 95°C for 30 seconds, annealing primers at 50°C for 30 seconds, and extending amplicons for one minute at 72°C. Sequences were generated on a CEQ 8000 capillary electrophoresis system, using a CEQ DTCS Quick Start Kit and the manufacturer's suggested protocols (Beckman Coulter, Fullerton, CA).

Experimental rearings were carried out between January and March of 2005. To minimize the loss of genetic variation during this period, the population was expanded to three cages of more than 100 individuals, from which 20–50 migrants were transferred as pupae to the other cages each generation. Generations were allowed to overlap until the cage required cleaning, which was done monthly while the next generation was in the juvenile form.

Cages of adult flies were provided water and honey *ad libitum*. Beef liver was supplied as a protein source one day prior to oviposition. On days that eggs were collected, fresh liver was placed into a cage in the late morning to mid afternoon. Cages were checked every 15–30 min until oviposition was observed. Approximately 250–1000 eggs (1–4 egg masses) were removed one h after the first observation of oviposition. The egg masses were immediately transferred to fresh liver and placed into a 1-liter glass canning jar (Alltrista, Muncie, IN), with a breathable cloth screwed on as a lid. Jars were placed into a temperature-controlled incubator at 25ºC (+/-0.5°C) with a 12:12 h light and dark cycle. A beaker filled with water was kept in the incubator, which provided a relative humidity of 25% (+/- 4%).

Several treatments were examined to assess the influence of rearing variables on the development time of specific immature stages, and on total immature development time (Table 1). These considered the freshness of food, moisture of food, type of pupation substrate used, orientation of the substrate with respect to food, transfer of larvae to fresh pupation substrate, and destructiveness of sampling. The influence of meat freshness was tested by providing cohorts with 40g of liver every day (fresh meat daily or FMD) or 120g of liver every third day (no fresh meat daily or NFMD). Paper towel treatments received fresh meat daily, which was placed on a moist paper towel (FMDPT). The influence of pupation substrate was examined by providing either clean sand (Fairmount, Wedron, IL) or vermiculite (Therm-O-Rock West, Chandler, AZ) to jars containing postfeeding third instars. The influence of food orientation with respect to pupation substrate was tested by either placing meat on top of the substrate at the egg stage, or placing the substrate on top of meat when larvae reached the postfeeding third instar stage. Fresh pupation substrate was tested by removing 125 postfeeding third instars from individual cohorts and placing them into a jar with 500ml of fresh pupation substrate. The

transfer treatments were taken from cohorts with far more that 300 individuals in the jar, meaning larval density was much greater in untransferred than transferred treatments. Destructive sampling was assessed by permanently removing or not removing 12 individuals from a cohort each day.

Experimental cohorts were checked approximately every twenty-four h, except jars with eggs, which were checked every half hour until they hatched, and pupae, which were observed throughout the day until eclosion occurred. Length measurements were taken throughout larval development, incorporating the 12 most mature larvae in all treatment groups (either the largest maggots or postfeeding maggots lacking blood in their crops). Ruler-measured lengths of the maximum body extension (to the nearest 0.5 mm) were determined using a stereomicroscope for first instars (due to their small size) or by eye for all other stages. Advances in developmental stage were recorded to the closest 15 minutes, however given that most animals were observed once per day, development time variation of less than one day was indistinguishable from sampling time variation. All experiments were conducted in the same temperature controlled incubator, with jars rotated within the incubator daily.

Development of larvae on mammal carcasses was performed using three Sprague-Dawley rats from breeding colonies at MSU, sacrificed by CO_2 asphyxiation within two days of egg placement on the body. The rats weighed approximately 500g and were in excess of the feeding needs of individual cohorts (larvae utilized approximately half of the carrion before the postfeeding stage). An egg mass collected in the manner detailed above was placed along the mouth of the rat. Rat carcasses were set in an open plastic bag, which was placed into a styrofoam container with an opening cut from the lid. A screen was fitted between the container and the lid to prevent escape of postfeeding larvae. Animals were reared at 25ºC (±0.5°C) and

25% (±4%) relative humidity, with maggot length and the duration of developmental stages recorded as above. Larvae from rat treatments were transferred to sand substrates to pupate.

Statistical Analyses.

Owing to unbalanced data (Table 1), MANOVA could not be used, thus analyses of variance were examined using Type III ANOVAs (Scheiner and Gurevitch 2001). This approach removes the variance from variables other than the one of interest, and compares the variance remaining to the dependent variable. ANOVA and regression statistics were performed with the R statistical package (R Development Core Team 2004) at a < 0.05 significance.

Development times in hours and accumulated degree-days (ADD), including standard deviations, were calculated for every significant treatment type and for rat cohorts. ADD was calculated using a base temperature of 10°C.

Graphs of larval growth were produced using the R statistical package. Curves were plotted by non-linear quantile regression using smoothing parameters that yielded curves comparable to published data from Greenberg (1991), Wells and Kurahashi (1994), and Grassberger and Reiter (2001). Treatments in the comparisons include FMD cohorts that were transferred to fresh pupation substrate, FMDPT cohorts that were transferred to fresh pupation substrate, and NFMD cohorts that were not transferred to new pupation substrate. The plots included average and 95% confidence intervals, from the day flies hatched until the first day pupae appeared, which were then compared to averages of larval growth on rats. Data from Grassberger and Reiter (2001) were also compared to larval development on rats, as that study included growth at 25ºC. For these analyses a locally weighted sum of squares (lowess) curve was plotted through the estimates using R.

Results

Species Identification.

Morphological identification of flies indicated that all were *L. sericata.* To confirm identification, a 798 base pair mitochondrial *cytochrome oxidase 1* sequence (NCBI accession number DQ062660) was obtained from a collected adult fly. A BLAST search showed it was identical to a *cytochrome oxidase 1* sequence from a *L. sericata* population in Ontario, Canada (accession number L14947). The closest 13 NCBI gene sequences were from *L. sericata*, with a maximum difference of 4 base pairs (<1%), confirming the species identification.

Developmental Plasticity.

The pre-pupation period for this fly population (reared at 25°C) ranged from 145–264.5 h (6–11 days), while the duration of egg to adult was 329–505.5 h (14–21 days), with all data given in Appendix 1. Throughout the experiment replicate treatments followed synchronized growth trajectories during the feeding stages, with a small number of individuals lagging. In contrast, postfeeding larvae within a treatment advanced to pupation gradually over a week. Eclosion took place over a week also.

Development times for stages and treatments are given in the Appendix and are summarized in Table 2 (using both hours and ADD). Linear models showed that development among treatments did not exhibit statistical differences in the shortest stages—the egg or the first two instars (a single exception is detailed below)—nor did these stages significantly influence total development time (data not shown). In contrast, the feeding portion of the third instar ($F=18.52$, df=1, $P=0.00013$, $R^2=0.35$), the postfeeding stage of the third instar ($F=27.67$, df=1, $P<0.0001$, $R^2=0.44$), and pupation ($F=53.59$, df=1, $P<0.0001$, $R^2=0.62$) significantly affected overall development times.

Substrate type and its placement had no significant effect on development during any stage. Other treatments examined (Table 1) significantly impacted development time (Figure 1 and Table 2), while the stage at which that impact occurred differed. FMD accelerated development compared to treatments that received supplements every third day during the feeding portion of the third instar ($F=12.19$, $df=1$, $P=0.0015$), although it was also a significant variable in the duration of the second instar ($F=8.336$, $df=1$, $P=0.0072$). Accordingly, the two treatment types that developed in 14–16 days were FMD. FMDPT also resulted in faster growth during the feeding portion of the lifecycle compared to treatments without paper towels ($F=206.8$, $df=1$, $P<0.0001$). Moist paper towels were not necessary for the most rapid overall development, given that the fastest recorded time from egg to eclosion was from a FMD transferred treatment [329 h (cohort 14 in Appendix 1)], however they promoted consistently faster development (Figure 1). Once feeding ceased, the moisture of food did not contribute to developmental variation (postfeeding third instar $F=0.8439$, $df=1$, $P=0.37$ for FMD and $F=1.677$, $df=1$, $P=0.21$ for FMDPT), however transferring larvae to fresh substrate significantly shortened the amount of time spent as postfeeding third instar larvae ($F=17.59$, $df=1$, $P=0.00022$). The results indicate that handling larvae during the study did not impede development.

Destructive sampling did not influence larval stages, but significantly increased the pupal stage ($F=49.13$, $df=1$, $P<0.0001$). Finally, variables were assessed together to determine their relative influence on total immature development. Each had significant effects on total development time (FMD: $F=4.644$, $df=1$, $P=0.039$; FMDPT: $F=8.019$, $df=1$, $P=0.0079$; Transfer to fresh substrate: $F=4.454$, $df=1$, $P=0.043$; Destructive sampling: $F=26.14$, $df=1$, $P<0.0001$).

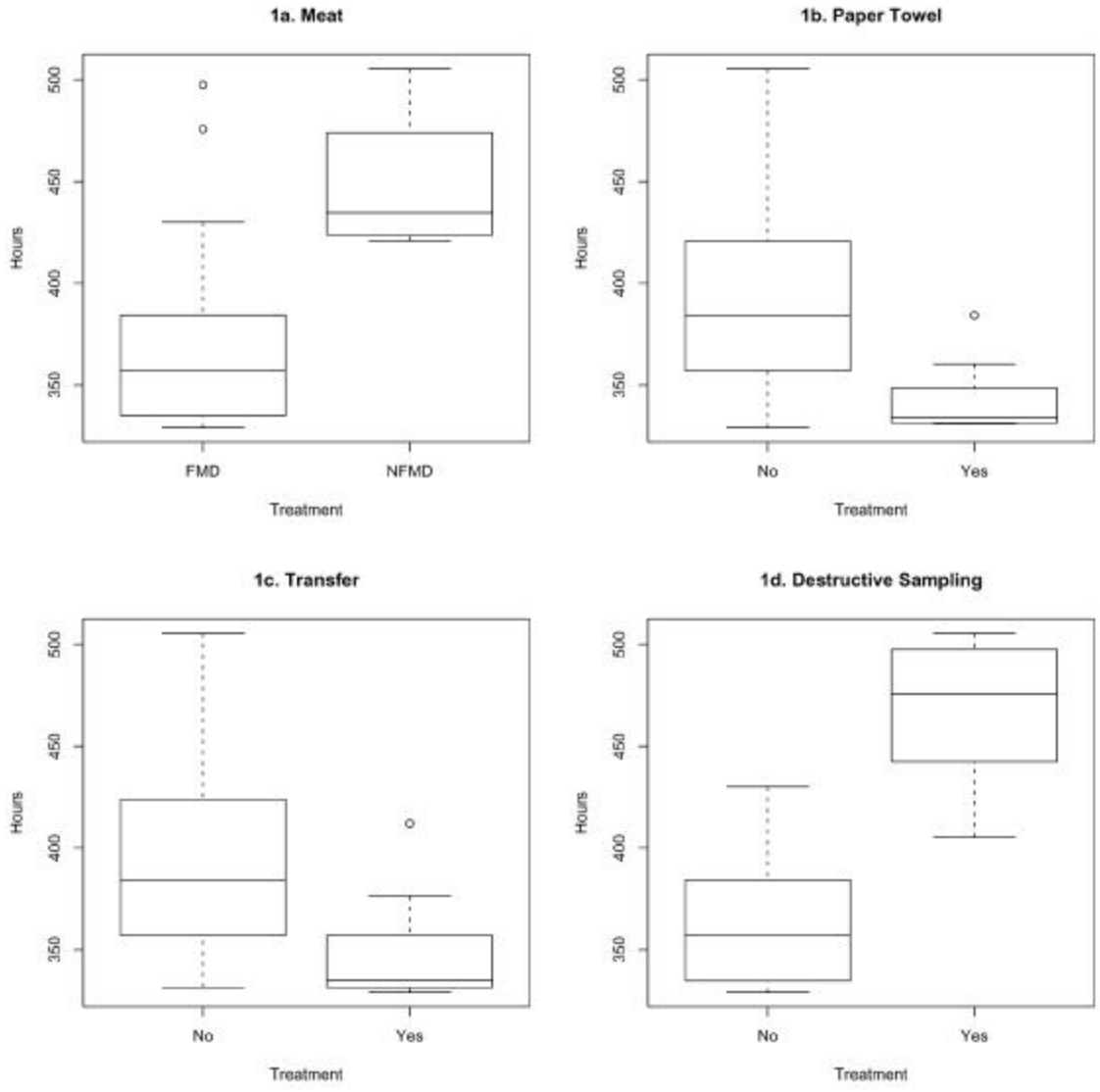

Figure 1. Developmental variation among *Lucilia sericata* cohorts by treatment type. Boxplots of total development time (h) for each of the 37 liver-fed cohorts. The line within the box represents the median development h, the box represents the development times between the 25th and 75thpercentiles, and the 'whiskers' (outer-most lines) represent the 5th and 95th percentiles. 1a: fresh meat daily or no fresh meat daily (FMD vs. NFMD); 1b: paper towel (moist paper towel placed under meat); 1c: transfer: transfer of larvae to fresh substrate for pupation; 1d: destructive (removal of 12 individuals each day). Note: treatments were in combination with other treatment types (Table 1) that had significant effects on development time. For instance, the two outliers in the FMD boxplot (1a) are those that were also destructively sampled.

Development on Carrion.

The pre-pupal growth of larvae on rats was compared to the statistically significant experimental treatments, as well as growth observed by Grassberger and Reiter (2001) (Figures 2 and 3). The results displayed in Figure 2 show tat the shape and rate of larval growth curves for FMDPT treatments most closely matched the three cohorts reared on rats.

Figure 3 displays the growth of larvae during the first three days of development, when growth rate is relatively constant. A linear regression demonstrated different rates of growth among treatments, which were 0.20 mm/hr, 0.10 mm/hr, 0.12 mm/hr, 0.21 mm/hr, and 0.23 mm/hr, for Rat, NFMD, FMD, FMDPT, and Grassberger and Reiter (2001) respectively, with R^2 values of 0.92, 0.77, 0.90, 0.95, and 0.99. The regression model showed that length varied significantly with age ($F=7099$, $df=1$, $P<0.0001$), while the effect of treatment types on length was also statistically significant ($F=281.8$, $df=4$, $P<0.0001$), as was the interaction between age and treatment type ($F=155.0$, $df=4$, $P<0.0001$).

Figure 4 compares the development of the flies reared on rats to development of liver-fed treatments in this study. Cohorts on rats developed in a manner that was most similar to the observed maximal development of liver-reared flies (i.e., FMDPT and some FMD treatments), with development times between 333 and 337 h (about 14 days). Further, growth on rat carcasses was much less variable than the growth of liver treatments.

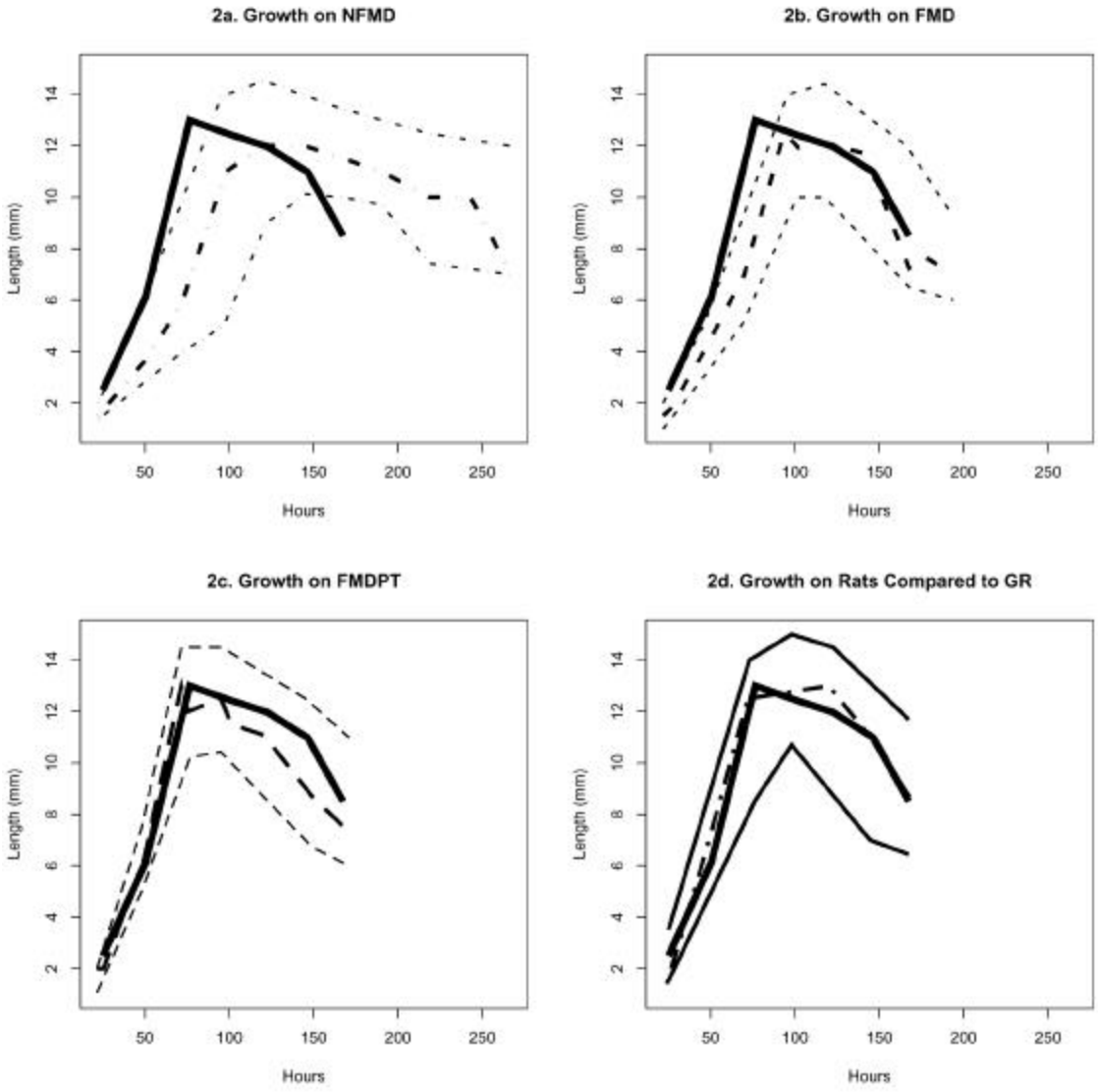

Figure 2. Growth curves of *Lucilia sericata* on liver versus growth on rat carrion. Non-linear quantile regression curves created from the lengths of maggots in daily collections of each treatment type. 2a: meat added every 3rd day, no moist paper towel, larvae were not transferred to fresh substrate to pupate; 2b: fresh meat daily, no moist paper towels, larvae transferred fresh substrate to pupate; 2c: fresh meat daily, moist paper towel used, larvae transferred to fresh substrate to pupate; 2d: the locally weighted sum of squares curve of data estimated from Grassberger and Reiter (2001) plotted against larval growth on rat carrion. Numbers of cohorts plotted for each treatment were 3, 4, 6, and 6 for Rat, NFMD, FMD, and FMDPT respectively. The solid line on each curve is the 50th percentile plot from cohorts raised on rats. Treatments are shown as dashed lines, with the thicker dashed line representing the 50th percentile and the thinner lines representing the 97.5th and 2.5th percentiles (95% confidence intervals). Confidence intervals for the rat cohorts are present in 2d.

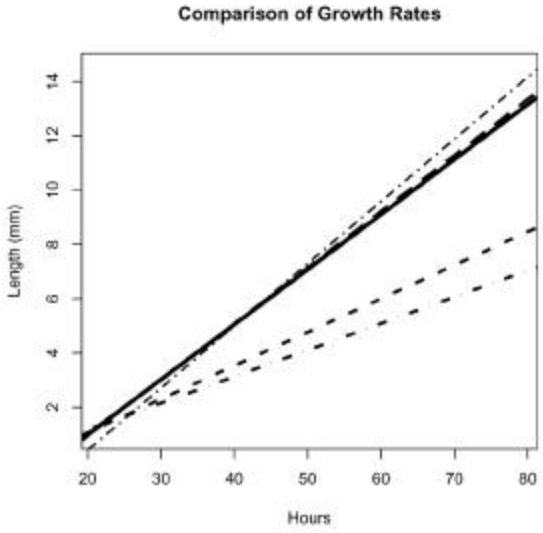

Figure 3. Linear growth of *Lucilia sericata* on liver versus growth on rats. Regression lines of the same treatments displayed in Figure 2, for the first three days of growth—the linear phase of development. Line types used to indicate treatments are the same as in Figure 2.

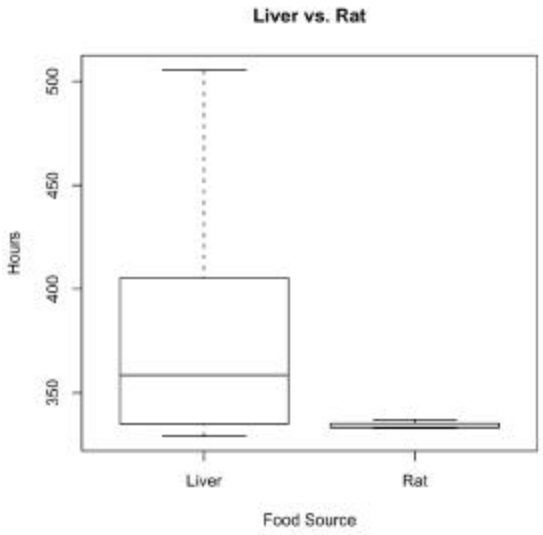

Figure 4. Development times of *Lucilia sericata* cohorts raised on liver versus rat carrion. Comparison of total development hours produced by the 37 liver-fed cohorts in this study to the development of the three rat-fed cohorts. Development time on rats was much less variable than growth on liver, with a development time most similar to the fastest growing liver-fed cohorts. Boxplot design is as in Figure1.

Discussion

Environmental Components of Variation in the Development of *L. sericata.*

The green blow fly is a widely distributed species of great forensic importance. Several authors have examined different fly populations reared under various environmental conditions, and perhaps not surprisingly, the developmental times differ from one another, with Kamal (1958), Greenberg (1991), and Grassberger and Reiter (2001) estimating faster minimum development times than Anderson (2000). This variability could result from genetic differences among populations, but could equally result from dissimilarity in the conditions under which the animals were reared. Further, none of the authors compared the laboratory growth of flies to that on actual carcasses. In the current study, designed to estimate variation in developmental rates resulting from environmental differences, a single population of *L. sericata* was grown under laboratory conditions that mimicked those used in the earlier studies, and these treatment were compared to larval development on carrion.

Given the minimum development times of the treatments detailed here, the fastest fell within the standard errors for *L. sericata* reared at 22ºC by Greenberg (1991) and Grassberger and Reiter (2001), and is close to the mode reported by Kamal (1958), which is a common forensic entomology resource. Likewise, the slowest minimum development time for flies in this study was longer than the developmental minimum at 23.3º C found by Anderson (2000). This indicates that environmental variation alone can potentially explain all differences in developmental rates detailed in earlier studies.

Results of these experiments demonstrate that variation in food moisture and pupation substrate have a significant influence on the growth of *L. sericata*; variation in rearing conditions generated a developmental difference of up to 7.4 days. Most notably, treatments designed to

maintain meat moisture during feeding shortened the development of larvae. FMDPT treatments significantly shortened the feeding portion of third instar larvae, and produced a much smaller developmental range (Figures 1 and 2). These results accentuate the importance of considering food moisture when rearing fly larvae. Grassberger and Reiter (2001) provided larvae with fresh liver daily, resulting in a similar growth rate at 25ºC. Other studies have included moist sawdust, paper towels, or wood chips underneath meat (Kamal 1958, Goodbrod and Goff 1990, Anderson 2000), which would be expected to hold moisture. Interestingly, moist paper towels changed the lifehistory table of FMD treatments toward the Greenberg (1991) estimate of third instar duration, which is approximately one day shorter than that of Grassberger and Reiter (2001). Unfortunately, Greenberg's (1991) report was vague about how flies were raised so it is unclear what other factors could be involved, but food moisture may play a role in the differences in third instar development time observations between these authors.

Transferring postfeeding larvae to a fresh substrate for pupation significantly shortened the time spent at this larval stage. The postfeeding portion of blow fly larval development is generally variable (Wells and Kurahashi 1994) and *L. sericata* is exceptional among blow flies for wandering far from its food to pupate (Anderson 2000). This may mean that *L. sericata* searches for a specific set of environmental cues for pupation, making the postfeeding stage susceptible to disturbance. The conditions that produced the fastest growth in this study yielded a postfeeding stage duration of two to three days. Kamal (1958) provided sawdust with food, and observed a mode postfeeding duration of 90 h at 26.7ºC, with a minimum of 48 h and a maximum of 192 h. His mode observation is similar to untransferred treatments in this study, which lasted a day longer than transferred cohorts. Greenberg (1991) reported an average postfeeding time of 108 h at 22ºC while Grassberger and Reiter (2001) reported 94 h at 20ºC and

87 h at 25ºC (the temperature at which this research was conducted). With little information on rearing conditions described by Greenberg (1991), the shorter times reported in the latter study are hard to explain, but Grassberger and Reiter (2001) reared their flies with dry sawdust in jars, which may have resulted in the shorter average duration, given that the treatment seems similar to the transfer treatments in the research presented here.

There is little information in the literature that helps explain the developmental variability between transferred and untransferred postfeeding larvae found in the current study. Three plausible explanations for this phenomenon are density of individuals in each cohort, moisture differences between old and fresh substrates, and difference in odor between the treatments. Larval density seems unlikely to have had much influence on development time. Several treatments that were transferred to sand had larvae that had congregated on the substrate surface, and these densely packed cohorts still pupated in a timely manner. On the other hand, a lack of moisture and odor are both plausible agents behind the accelerated onset of pupation in transferred larvae. The sensitivity of larvae to moisture during feeding (outlined above) indicates that moisture is a potential cue for the cessation of feeding, with maggots actively searching out wet areas (tissues) while feeding, and reversing this behavior when heading towards pupation. Likewise, blow flies are attracted to odors associated with decay (Catts and Haskell 1990, Chaudhury et al. 2002, Hall et al. 2003), thus it might be advantageous to be attracted to putrefying odors during feeding, followed by a pre-pupation move away from such odors.

Destructive sampling was found to be unimportant in larval development, yet was the only significant variable affecting the duration of pupation. The delay in pupation most likely resulted from the elimination of the earliest individuals to form a puparium, which were

destructively sampled (removed) by necessity. Given these findings, studies of pupal development rates that require destructive sampling should consider its effects.

Other Potential Sources of Variation.

The data presented demonstrate the effects of differing rearing treatments on this population of *L. sericata.* It should be noted however, while most variation in growth existed among treatments, within-treatment variation was also observed. A portion of this could be explained by unmeasured environmental factors, as only a small number of rearing modifications were tested. Certainly, factors not considered in this study are likely to impact developmental differences.

Likewise, though environmental conditions were found to be highly significant in the development of *L. sericata,* genetic variation among fly populations used in different studies could potentially be just as important in understanding developmental variability. It is necessary to remember that each publication mentioned above outlined the development of flies that originated from a different ecogeographical region. There is precedence for population effects on the development of blow flies and several related species (Johnson and Schaffer 1973, Greenberg 1991, Oudman et al. 1991, Hoffmann and Harshman 1999, Parsch et al. 2000, Blanckenhorn 2002, Ames and Turner 2003, Feder et al. 2003). Genetic makeup is likely to affect other populations of blow flies, although these have been largely untested. Genetic differences, including potential interactions between genotype and environment, may be important sources of developmental variation when comparing populations of *L. sericata.*

Optimal Rearing Condition Using Liver and Growth on Rat Carrion.

One might expect that blow flies have evolved to develop fastest under natural conditions of carrion decomposition. If this is the case, the fastest growth rate obtained in laboratory

rearings would be expected to mimic the growth of flies living on carrion at the same temperature. In the current study, *L. sericata* development on rat carcasses was most similar to flies reared under high moisture conditions (Figure 4). This finding helps address concerns raised by Kaneshrajah and Turner (2004) and Clark et al. (2006) who observed a significant effect of tissue type on the growth of *C. vicina* and *L. sericata*, respectively. Kaneshrajah and Turner (2004) were critical of rearing flies on liver, as it seemed to delay development. This delay was similar to slower developing treatments observed on desiccated liver in the current study, suggesting that larval rearing should take place on non-desiccated substrates to best mimic growth on a corpse.

Applications to Forensic Entomology.

L. sericata development is plastic, at a level that alone could explain differences in the species' published developmental times. This finding highlights two important factors that need to be considered when estimating a PMI based on blow fly development. First, the discrepancies among development data sets can potentially be explained, *in toto*, by differences in laboratory rearing protocols used to develop such timetables. Accordingly, establishment of a common set of rearing conditions, which best relate to growth on carrion, is critical if direct comparisons are to be made among datasets, and if these data sets are to be used in legal proceedings. Second, because forensic entomologists use a quantitative trait (development rate) and decomposition ecology to make PMI estimates, researchers conducting studies on development time must aim to address the effects of both genetics and environment on their findings. By doing so, the forensic community can achieve a greater understanding of how important each of these factors is to forensic entomology.

A final consideration regarding entomological evidence involves its legal use in general. In the wake of judicial decisions that place a far greater emphasis on systematic analyses, known error rates, and statistical probabilities (see Daubert v. Merrell Dow Pharmaceuticals, 509 US 579 (1993), and KumhoTire Co. v. Carmichael, 526 US 137 (1999)), forensic scientists are under increasing pressure to conduct research, present legal analyses, and draw conclusions in a methodical and scientifically replicable way, while relying less on generalized knowledge and personal experience. The field of forensic entomology, although based on sound scientific principles, can currently be included among an assemblage of forensic disciplines that may be called into question with regards to repeatability and standardized techniques (see Saks and Koehler, 2005). Efforts to establish calliphorid laboratory rearing protocols that best portray fly development on cadavers, and to standardize those techniques for future research, are central to meeting the demands of Daubert and Kumho. Such endeavors are necessary if forensic entomological evidence is to be routinely accepted in courts of law.

Table 1. Treatment types for the 37 liver-fed cohorts

Meat	Destructive	Transfer	Substrate Under	Substrate	Paper Towel
FMD: 33	No: 32	No: 24	No: 33	Sand: 14	No: 25
NFMD: 4	Yes: 5	Yes: 13	Yes: 4	Vermiculite: 23	Yes: 12

Meat: Fresh meat daily (FMD) or not (NFMD). Destructive (sampling): 12 individuals removed from the cohort at each sampling time. Transfer: 125 postfeeding third instars were transferred to 500 mL of fresh pupation substrate. Substrate Under: food was placed on top of a substrate or at the bottom of an empty jar. Substrate: pupation substrate used. Paper Towel: received FMD placed on a moist paper towel.

Table 2. Average development times ± standard deviations in hours and accumulated degree-days* for significant rearing treatments of *Lucilia sericata.*

Treatment	Egg	1st Instar	2nd Instar	3rd Instar	Postfeeding	Pupa	Total
FMD	21.74±2.72	26.56±2.18	26.05±4.59	40.02±12.55	76.04±19.85	177.8±26	368.2±41.63
	(13.6±1.7)	(16.6±1.36)	(16.28±2.87)	(25.01±7.85)	(47.52±12.41)	(111.1±16.25)	(230.1±26.02)
NFMD	21.75±2.1	25.25±0.87	36.44±12.49	58.75±12.7	98.5±17.71	208.3±58.93	448.9±38.8
	(13.59±1.31)	(15.78±0.54)	(22.77±7.81)	(36.72±7.94)	(61.56±11.07)	(130.2±36.83)	(280.6±24.25)
Paper Towel	22.5±2.24	25.75±1.98	26.13±3.37	23.88±1.4	68.06±13.31	175.6±15.91	341.9±16.85
	(14.06±1.4)	(16.09±1.24)	(16.33±2.11)	(14.92±0.87)	(42.54±8.32)	(109.7±9.95)	(213.7±10.53)
No Paper Towel	21.38±2.77	26.74±2.13	27.68±7.59	50.76±6.12	83.46±21.87	183.8±36.53	393.8±49.25
	(13.36±1.73)	(16.71±1.33)	(17.3±4.74)	(31.73±3.82)	(52.16±13.67)	(114.9±22.83)	(246.1±30.78)
Transfer	22.02±3	26.25±2.25	25.87±2.84	37.6±13.37	59.88±15.33	175±12.2	346.6±24.35
	(13.76±1.88)	(16.41±1.4)	(16.17±1.78)	(23.5±8.36)	(37.43±9.58)	(109.4±7.62)	(216.6±15.22)
No Transfer	21.59±2.46	26.51±2.07	27.89±7.79	44.45±13.58	88.53±15.62	184.4±37.78	393.4±50.04
	(13.5±1.54)	(16.57±1.3)	(17.43±4.87)	(27.78±8.49)	(55.33±9.76)	(115.3±23.61)	(245.9±31.2)
Destructive	21.7±1.89	26.65±2.33	28.7±10.22	52.85±9.8	92.2±9.72	243.2±41.23	465.3±41.5

Treatment	Egg	1st Instar	2nd Instar	3rd Instar	Postfeeding	Pupa	Total
Destructive	(13.56±1.18)	(16.66±1.46)	(17.94±6.39)	(33.03±6.12)	(57.63±6.08)	(152±25.77)	(290.8±25.94)
Not Destructive	21.75±2.75	26.38±2.11	26.94±5.94	40.35±13.58	76.32±21.16	171.4±14.22	363.2±31.72
	(13.59±1.72)	(16.49±1.32)	(16.84±3.71)	(25.22±8.49)	(47.7±13.22)	(107.1±8.89)	(227±19.82)
Rat	19.67±1.04	30.67±1.53	23.5±1.73	24.33±0.58	60.67±12.29	175.7±11.58	334.5±2.18
	(12.29±0.65)	(19.17±0.95)	(14.69±1.08)	(15.21±0.36)	(37.92±7.68)	(109.8±7.24)	(209.1±1.36)

*Accumulated degree-days, using a base temperature of 10ºC. Values displayed parenthetically.
3rd Instar: the feeding portion of the stage.
Postfeeding: the non-feeding portion of the 3rd instar.

Appendix 1. Treatments and duration of the immature life cycle and individual stages from all cohorts of *Lucilia sericata*.

Cohort	Meat	Destructive	Transfer	Substrate Under	Substrate	Paper Towel	Egg
1	NFMD	No	No	No	Vermiculite	No	21.5
2	NFMD	Yes	No	No	Vermiculite	No	22.5
3	FMD	Yes	No	No	Vermiculite	No	20
4	FMD	No	No	No	Vermiculite	No	24
5	FMD	No	No	Yes	Vermiculite	No	21
6	FMD	No	Yes	No	Vermiculite	No	24
7	NFMD	No	No	No	Vermiculite	No	19
8	NFMD	Yes	No	No	Vermiculite	No	24
9	FMD	Yes	No	No	Vermiculite	No	22.5
10	FMD	No	No	No	Vermiculite	No	25
11	FMD	No	Yes	No	Vermiculite	No	25
12	FMD	Yes	No	No	Vermiculite	No	19.5
13	FMD	No	No	No	Vermiculite	No	18.5
14	FMD	No	Yes	No	Vermiculite	No	18.5

Cohort	Meat	Destructive	Transfer	Substrate Under	Substrate	Paper Towel	Egg
15	FMD	No	No	Yes	Vermiculite	No	21
16	FMD	No	No	No	Sand	No	15
17	FMD	No	Yes	No	Sand	No	15
18	FMD	No	No	No	Sand	Yes	18.5
19	FMD	No	Yes	No	Sand	Yes	18.5
20	FMD	No	Yes	No	Sand	Yes	24
21	FMD	No	No	No	Sand	Yes	24
22	FMD	No	No	Yes	Sand	No	19
23	FMD	No	No	Yes	Sand	No	22
24	FMD	No	No	No	Sand	No	24
25	FMD	No	Yes	No	Sand	No	24
26	FMD	No	No	No	Vermiculite	Yes	22
27	FMD	No	Yes	No	Vermiculite	Yes	22
28	FMD	No	Yes	No	Vermiculite	No	21.75
29	FMD	No	Yes	No	Sand	No	23

Cohort	Meat	Destructive	Transfer	Substrate Under	Substrate	Paper Towel	Egg
30	FMD	No	Yes	No	Sand	Yes	21.5
31	FMD	No	No	No	Vermiculite	No	21.75
32	FMD	No	No	No	Sand	No	23
33	FMD	No	No	No	Sand	Yes	21.5
34	FMD	No	Yes	No	Vermiculite	Yes	24.5
35	FMD	No	Yes	No	Vermiculite	Yes	24.5
36	FMD	No	No	No	Vermiculite	Yes	24.5
37	FMD	No	No	No	Vermiculite	Yes	24.5
38	Rat	No	Yes	No	Sand	No	20.5
39	Rat	No	Yes	No	Sand	No	20
40	Rat	No	Yes	No	Sand	No	18.5

1st Instar	2nd Instar	3rd Instar (Feeding)	Postfeeding	Pupa	Total Hours	Total Days
26	47	69.5	100.5	162.5	427	17.79
24.5	25.25	48	98.25	224	442.5	18.44
30	21.75	47.25	94.5	284	497.5	20.73
27	28	45	98	162.25	384.25	16.01
29.5	22.75	49	99.25	187.5	409	17.04
27	28	45	48	163.25	335.25	13.97
26	47.5	47.5	119.25	161.5	420.75	17.53
24.5	26	70	76	285	505.5	21.06
26.5	23.75	52	91.5	189	405.25	16.89
25.25	23.75	51	118	187	430	17.92
26.25	23.75	51	94	192	412	17.17
27.75	46.75	47	100.75	234	475.75	19.82
28	23.5	52.25	95.25	140	357.5	14.9
28	23.5	52.25	43.75	163	329	13.71
25.5	22.5	53	93	160	375	15.63

1st Instar	2nd Instar	3rd Instar (Feeding)	Postfeeding	Pupa	Total Hours	Total Days
30.75	23.75	48	95.5	163	376	15.67
30.75	23.75	48	71.75	187	376.25	15.68
29.5	25.5	25	69	164	331.5	13.81
29.5	25.5	25	48	185	331.5	13.81
26.75	28	21.5	72.25	162.5	335	13.96
26.75	28	21.5	72.25	162.5	335	13.96
27.75	24.75	47.75	93.75	188	401	16.71
28	25	47	72.25	162.75	357	14.88
27.25	24	48.25	65	168.5	357	14.88
27.25	24	48.25	65	168.5	357	14.88
24	29.5	23.5	71.75	162.25	333	13.88
24	29.5	23.5	71.75	162.25	333	13.88
24	28.25	50.25	47	163.25	334.5	13.94
23.5	28.25	50.75	46.75	167.75	340	14.17
24.25	29.75	25.75	70.5	188.5	360.25	15.01

1st Instar	2nd Instar	3rd Instar (Feeding)	Postfeeding	Pupa	Total Hours	Total Days
24	28.25	50.25	65.25	166.5	356	14.83
23.5	28.25	50.75	94.25	164.25	384	16
24.25	29.75	25.75	70.5	212.5	384.25	16.01
25	22	23.5	50.25	185.75	331	13.79
25	22	24	49.5	186	331	13.79
25	22	23.5	95.5	168	358.5	14.94
25	22	24	75.5	167.5	338.5	14.10
29	22.5	24	68.5	172.5	337	14.04
31	25.5	24	67	166	333.5	13.9
32	22.5	25	46.5	188.5	333	13.88

The minimum development times of each stage and the minimum total development time for cohorts of *Lucilia sericata.* Also listed are the combinations of variables that each cohort experienced. All times are reported in hours (to the closest quarter hour) except the total development time, which is reported in hours and days. Minimum development times ranged from 329 to 505.5 hours for liver-fed cohorts (Cohorts 1–37) and from 333 to 337 hours for rat-fed cohorts (Cohorts 38–40). Labels are as in Table 1.

The Use of Generalized Additive Models in Analyzing Forensic Entomological Data (The following section is under review in the Journal of Forensic Sciences)

Daubert, et al. *v*. Merrell Dow Pharmaceuticals (509 US 579 (1993)) was a pivotal ruling for forensic scientists, in which the US Supreme Court declared that the Federal Rules of Evidence (particularly Rule 702), and not Frye (Frye *v*. United States (293 F. 1013, 1014, D.C. Cir. (1923)), were the standard for scientific evidence and expert testimony. In doing so, the High Court placed the burden of assessing the validity—and thus admissibility—of scientific evidence on the trial judge, based on five main criteria: Has the technique in question been tested; Do standard operating procedures (SOPs) exist for the technique; Has the technique been subjected to peer review and publication in the appropriate literature; Is the technique widely accepted by the relevant scientific community; and finally, What is the known or potential error rate of the technique? DNA-based evidence has set the 'gold standard' for meeting Daubert requirements, largely satisfying all of them. In contrast, many of the forensic sciences and resultant expert testimony are based on practitioners' training and experience, often with little consideration for SOPs, method testing, potential error rates, or publication, even when the technique is generally accepted. As an example, the National Institute of Justice recently posted a solicitation for the study of fingerprints/friction ridges, though certainly this method of identification is extremely well-established. Other areas of forensic science fare far worse (Saks and Koehler 2005).

Forensic entomology falls between these extremes. The predictable growth of carrion-feeding flies has long been used to estimate the time a body has been exposed to insects, and thus to estimate a post mortem interval (PMI). Using larval size and developmental stage to approximate age is well supported by research and observations in developmental biology, and

this forensic technique is widely described in the scientific literature (e.g., Greenberg 1991, Grassberger and Reiter 2001). Likewise, countless legal rulings have assured its admissibility, just as countless juries have been guided by entomological testimony. However, scientists have reported different growth rates for immature flies (Kamal 1958, Greenberg 1991, Wells and Kurahashi 1994, Anderson 2000, Grassberger and Reiter 2001) and court qualified experts have come to incongruent conclusions about a PMI based on the same entomological evidence, depending on which growth data were utilized (e.g., California *v*. Westerfield, CD 165805 (2002)). This problem stems, at least in part, from a general failure to develop SOPs, and also from not fully considering the amount of variation present in larval growth (or more precisely, to account for error rates inherent in estimates of larval age), two of the major tenets of Daubert. The difficulty in estimating error is exacerbated by the fact that blow flies grow in a non-linear fashion and have variable size distributions at different ages, unequally affecting age estimates of developmental stages (Wells and Lamotte 1995).

The research presented here was designed to investigate the variability that occurs in larval and pupal growth of blow flies in order to discern which of a suite of variables have the largest influence on estimating age, and to explore the possibility of placing confidence intervals around juvenile age estimates. Using three regional strains of the blow fly *Lucilia sericata* (Diptera: Calliphoridae) (Meigen), collected in California, Michigan, and West Virginia, a data set containing linear (developmental stage, strain, rearing temperature) and non-linear (length and weight) measures was established. Generalized additive models (GAMs) were developed taking these variables into account, examining the level to which each influenced/predicted the percent of immature fly development (Hastie and Tibshirani 1990, Wood 2006). Similar GAMs have already been used to assess the effects of cadmium on the growth of *L. sericata* cohorts

(Moe et al. 2005), and were assessed here for their potential use as tools in predicting blow fly development percent. The utility of a model was then tested on an independent data set (larvae reared on rat carcasses), focusing on developmental stage and length. GAM predictions of larval development percent were plotted against true age to assess the error of the predictions and to define confidence intervals for these estimates.

Materials and Methods

Species Identification.

Wild *L. sericata* were collected in California (CA), Michigan (MI), and West Virginia (WV), from the UC Davis campus in June of 2005, the Michigan State University campus starting in May 2005 (which were provisioned with new flies occasionally throughout the summer), and from the West Virginia University campus in August of 2005. Adult individuals from each strain were identified by key (Hall 1948 and Gorham 1987), with independent confirmations, and through mitochondrial cytochrome oxidase 1 gene sequencing (Tarone and Foran 2006).

Growth Experiments.

Cohorts of flies were raised in a round robin design, in which CA and MI were reared in one block, followed by CA and WV, and WV and MI, between 9/1/05 and 10/24/05. Flies ranged from two to five generations removed from their natural population. Cohorts were initiated by placing fresh liver into the cages of adult flies, which was checked regularly for eggs. When oviposition occurred the time was recorded and meat and eggs were removed 1 h later. Cohorts were placed in either 20±0.5ºC or 33.5±1.8ºC incubators under a 12:12 h light cycle at 25±5% relative humidity. Incubator temperature fluctuation was noted using a HOBO data

logger (Onset Computer, Bourne, MA). Eggs were transferred to fresh liver, which was placed on a moist paper towel in 1 L jars, covered with a breathable fabric lid, based on rearing conditions previously found to best mimic those on carrion (Tarone and Foran 2006). Cohorts were given fresh liver daily until postfeeding third instars were observed, at which point 250 individuals (33.5ºC treatments) and 375 individuals (20ºC treatments) were transferred in batches of 125 to 1 L jars containing 500 mL of fresh sand as a pupation substrate.

Length and weight of 2559 larvae/pupae were recorded, starting approximately 24 h after eggs were laid. Length was measured with a ruler based on the furthest extension of a larva to the nearest ½ mm. Wet weight of live individuals was measured on a Cahn 27 Automatic Electrobalance (Cahn Instruments, Cerritos, CA) to the closest 1/100 mg. Developmental stage was assessed by observing feeding larvae microscopically, by visible crop length and migrating behavior for postfeeding larvae, and puparium formation for pupae. Ten larvae were removed from a cohort and measured/weighed, twice daily (in the morning and late afternoon). Ten pupae were collected once daily and measured/weighed; 5 individuals were collected if less than 10 were available.

Earlier research showed that the destructive sampling of pupae delayed the appearance of adults (Tarone and Foran 2006). To account for this, pupal age was calibrated to the day of pupation. This means that pupal samples were assessed in groups that pupated within 24 h of each other (i.e. 0–1, 1–2, 2–3, etc. day old puparia) with the minimum development time for pupation being the minimum development time for any individual within a collective group of pupae.

Forensic entomologists generally assess fly growth progression using a measure of relative age, allowing them to take into account the substantial influence of temperature on

development. Given that multiple variables had the potential to affect immature fly growth rates in the current research, including understood (e.g., temperature) and questioned (e.g., fly strain) factors, a method that would allow growth progression to be compared directly among all flies was required. Development percent, or the relative (developmental) age of an individual, was used to assess the extent to which a fly had progressed towards maturation (eclosion). This measure, often used for relative developmental comparisons (e.g., Rogina and Helfand 1995, Rogina et al. 1997, Anderson 2000), permitted individuals at all points in development to be compared, which would be impossible if, for instance, temperature and fly strain varied in their influence on growth. Development percent was calculated by determining the age in hours of an individual, then dividing the age by the minimum total development time of that experimental replicate. As an example, if an individual was sampled 100 h after oviposition and the minimum development time for the replicate was 285 h, then the individual was considered 35% developed.

The laboratory growth of larvae on rats have been described previously (Tarone and Foran 2006) and differed from the measured cohorts primarily in food source and temperature (25ºC). Three cohorts of Michigan *L. sericata* larvae were reared on rat carcasses and the developmental stage and length of twelve individuals were recorded daily from each cohort through the first day that puparia were observed. These data were used to predict age. The ethical guidelines of the Michigan State University Laboratory Animal Resources unit were followed, adhering to IACUC requirements.

Statistical Analyses.

GAMs were developed using the mgcv library in the R statistical package (R core development team 2004). The models use likelihood statistics to predict a value (e.g., age) based

on various input data. GAMs relate non-linear data such as fly length and weight to the predicted value (e.g., development percent) using smoothed, non-linear mathematical functions (Hastie and Tibshirani 1990, Moe et al. 2005, Wood 2006). In this manner, the relationship of two non-linear variables to each other can also be included in GAMs (Wood 2006), so a length-by-weight term was also evaluated. Distributions must be applied to the functions used to make predictions in a GAM, which is done through a link function. Based on the results of residual plots produced for the models, a gamma distribution (instead of a normal distribution) with a log link function was most appropriate for the models evaluated. Diagnostic plots were compared among models in order to confirm the validity of distributional assumptions in a model and to compare the predicted versus true age. The first plot was a quantile-quantile graph of modeled data versus data from samples. If the assumptions of a model are correct, this line is straight. The next plot was a graph of residuals against predictions. The data should be evenly distributed above and below zero, with no difference in residuals along the linear predictor axis; unevenly dispersed residuals indicate that the assumed data distribution in the model is inaccurate. The third plot was a comparison of the distribution of residuals, which should appear as a bell curve (most error is small, with rare instances of larger error). The final plot was a graph of true (response) versus predicted (fitted) values for all data used to construct the model. For simplicity's sake this will be referred to as the Y = X line, or Y (predicted age) = X (true age). The most precise models have all predicted age values clustered close to the Y = X line, with no gaps in the line. A gap in predictions results in an aging inaccurary because an individual of an age found in a gap will necessarily be predicted as either older or younger than it actually is. More detailed information on GAM can be found in (Hastie and Tibshirani 1990, Moe et al. 2005, Mansson et al. 2005, Wood 2006).

Models generated several statistics. For linear models the statistic used to explain how closely data match a model is R^2; as length and weight data are non-linear the apposite statistic for GAMs is the percent deviance explained (Wood 2006). Degrees of freedom or estimated degrees of freedom (a non-linear equivalent) were determined, as was a P-value, which was based on the likelihood of a variable being predictive of age. P-values in GAMs are considered estimates because likelihood statistics do not yield actual P-values, but do provide values that are similar and can be used to estimate the more familiar statistic. These estimates can vary by up to two times the actual P-value (Wood 2006), thus terms were not considered significant unless P-values were less than 0.025. Additionally, multiple variables were included in some models, requiring a Bonferroni correction that resulted in a significance threshold of $P<0.0042$. Given the inherent inaccuracy of estimated P-values, they were only used to identify informative terms or terms that were candidates for removal from a model owing to intermediate or non-significant P-values. The inclusion or removal of a term, however, was ultimately decided by the statistic used to compare models: the generalized cross validation (GCV) score, which is an information criterion that is lower for better models (Wood 2006).

Six terms were used to develop models: fly developmental stage, length, weight, length-by-weight, strain, and temperature. Stage, strain, and temperature were considered linear variables, and length, weight, and the two plotted against each other were non-linear. This resulted in 63 possible models, hence only a subset is presented here. The first six models examined each variable by itself, while the remaining 12 combined variables to assess improvements gained (as measured by a decrease in GCV) from including specific terms. Developmental stage was considered the primary variable, as all forensic entomologists include this in PMI predictions. Body size is also often incorporated into PMI estimates, thus length and

weight were added to several models, as well as being examined in combination. Next, the influences of strain and temperature were tested through inclusion with the more familiar variables (stage, length, weight). Similarly, since length-by-weight is a somewhat novel measure it was evaluated in combination with the three standard variables, and then with all variables.

Finally, a GAM incorporating the standard variables used to age flies in forensic entomological enquiries, developmental stage and length (Kamal 1958, Greenberg 1991, Anderson 2000, Grassberger and Reiter 2001, Tarone and Foran 2006), was tested against an independently derived data set. The model-based predictions of larval development percent for three previously collected fly cohorts raised on rats were plotted against their true development, comparing them to the predicted 95% confidence intervals for the model (precision) and the Y = X line (accuracy). Confidence intervals were superimposed over the predictions made for rat cohorts (using the quantreg library in R) by plotting locally weighted sum of squares curves through the 97.5^{th} and 2.5^{th} percentiles.

Results

Species Identification.

Flies collected from the three states were identified as *L. sericata* based on both visual verification, visual confirmation by an independent entomologist, and cytochrome oxidase 1 sequence data (accession numbers DQ868503, DQ868523, and DQ868524 for CA, MI, and WV respectively). Sequences obtained from the CA strain, the MI strain, and the WV strain were 428 and 227 non-overlapping base pairs, 774 continuous base pairs, and 776 continuous base pairs in length, respectively. BLAST results for the sequences showed the closest match for all

was to *L. sericata*, with 100 % similarity to at least one other *L. sericata* sequence. The next closest species match was *L. cuprina* with a 98% to 99% similarity (5–8 base pairs difference).

Immature Development.

Figure 1 depicts a plot of fly length against percent juvenile development. The feeding portion of the lifecycle makes up the initial 25%, and shows a linear increase in length. The postfeeding third instar, where body size decreases and variation in size increases, is found from approximately 25–50%. The relatively unchanged second half of the plots is the pupal stage. Weight results displayed the same pattern (data not shown), and both demonstrated that the distribution of sizes in the feeding stages was much smaller than it was in postfeeding third instar larvae and pupae. Minimum and maximum development percents for each stage of development were: First instar = 5.5–11.0%; Second instar = 7.4–15.4%; Feeding third instar = 12.6–26.0%; Postfeeding third instar = 19.1–60.1%; and Pupa = 43.2–100% (Figure 2).

Size was influenced slightly, but significantly, by temperature and strain. CA individuals tended to be larger than MI, which were larger than WV (Figure 3). Differences in size among strains were not observed during feeding stages, but were observed once feeding ceased (Figure 3) as each strain initiated the postfeeding third instar at different points in development, resulting in variation in average pupal sizes. Also, growth at 20ºC yielded larger individuals on average than did growth at 33.5ºC, presumably due to a change in the relative rate of development for feeding larvae (Figure 3). Size differences caused by both strain and temperature were repeatable, though average differences were well within the variation observed for size traits (e.g., Figure 1), resulting in an overlap of body sizes among all strains and both temperature treatments.

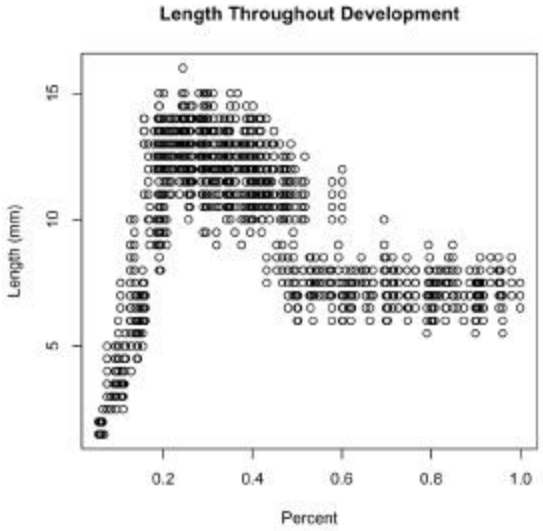

Figure 1. The lengths (mm) of 2559 immature *L. sericata* throughout immature development (percent of development values are on a 0–1 scale). Note the tight distribution of sizes during the earlier, linear growth phase compared to the more variable postfeeding third instar and pupal stages

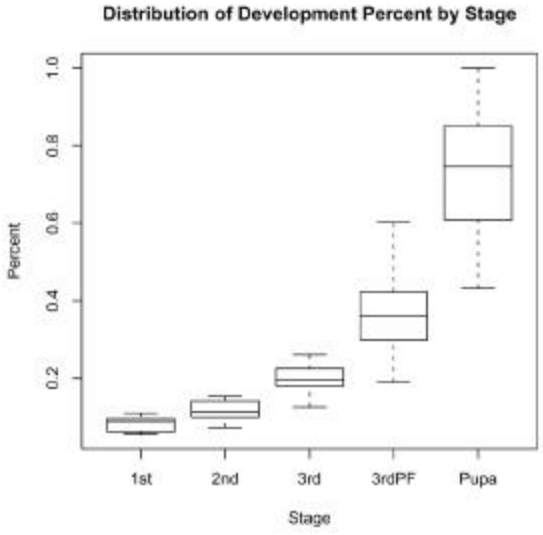

Figure 2. A plot of the distribution of development percents for individuals at each developmental stage. As development progressed, the proportion of the lifecycle spent in a stage increased. 3rd indicates the feeding portion of the third instar; 3rdPF indicates the postfeeding stage of the third instar.

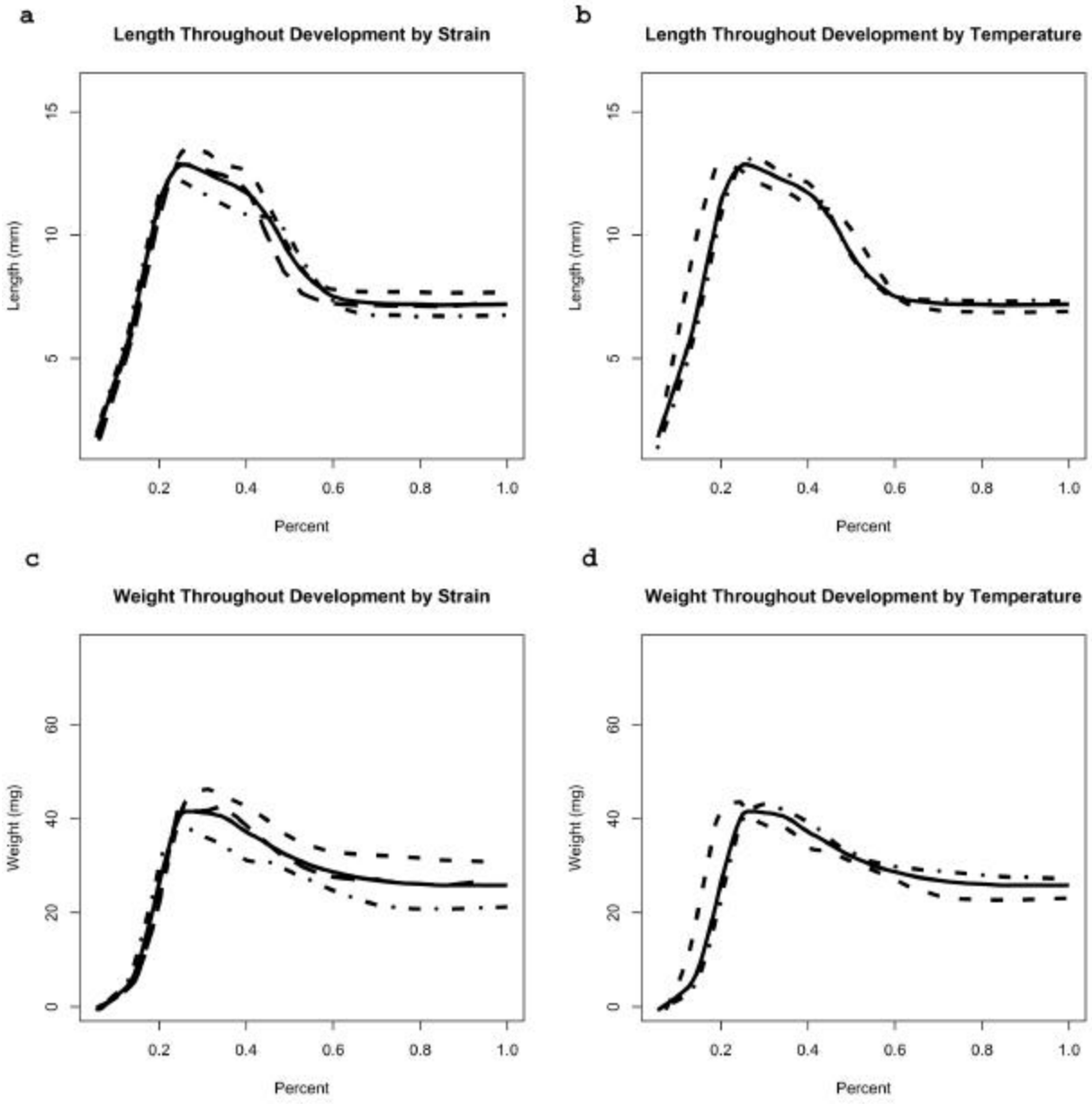

Figure 3. The lengths and weights of individuals throughout development from the 6 cohorts. Growth is compared by strain and by temperature. Solid lines represent the average for all strains or both temperatures. a) Length (mm) plots for each strain. The largest strain, denoted by the line with short bars and spaces, was CA, and the smallest strain, designated by the line with short bars separated by dots, was WV. The MI strain was close to the average size and is represented by the spaced line with long bars and short spaces. Less size variation existed during the feeding portion of the lifecycle (when size was increasing) than in the postfeeding and pupal stages. b) Length plots comparing growth at 20ºC versus 33.5ºC. Growth at 20ºC is represented by the spaced line with short bars separated by dots and 33.5ºC is represented by the line with short bars and long spaces. The higher temperature resulted in a growth curve that had a steeper slope during the linear growth phase of development; individuals from these treatments peaked in body size proportionally faster than cooler treatments, which resulted in smaller body sizes as

pupae. c) Weight (mg) plots for each strain. Comparisons among strains were as in (a). d) Weight plots for the two temperature treatments, with similar results as in (b).

Assessing Statistical Models.

All models demonstrated acceptable levels of error in the diagnostic plots (Figure 4a–4f), indicating that the use of a gamma distribution with a log link function was appropriate. A comparison of all models examined (Table 1) displayed the utility of GAMs to predict development percent when different variables were included. Stage was the single most informative variable (GCV = 0.045), while length and weight garnered less information (GCV = 0.126 and 0.144 respectively); all were statistically significant ($P < 0.0001$; Table 2). The length-by-weight term (model 4) provided an intermediate level of information in assessing development (GCV = 0.059). Temperature and strain were not significant predictors of age by themselves (models 5 and 6; GCV = 0.358 for both) and only provided useful information ($P < 0.0001$ and a decreased GCV) when combined with other variables (e.g., model 18). Predictions with length and weight yielded similar results to model 4, approaching, but not improving upon, the explanatory power of stage alone (model 12; GCV = 0.064). Any model that included stage and at least one body size measure explained 90.8–92.6% of the deviance in the data and GCV scores of 0.04–0.034, with the model that included all variables garnering the highest percent deviance explained and the lowest GCV.

All models were limited in predicting the ages of postfeeding third instars and pupae, generating artificially narrow age ranges. Gaps between stages were most dramatic in model 1 (developmental stage alone), wherein individuals were simply predicted to be the average age of that stage, although true ages were continuous. Inclusion of body size improved predictive precision in the early stages, but not for postfeeding third instars and pupae. As an example, in

model 10, which included developmental stage and length, postfeeding third instars that were 19.1–60.1% developed (Figure 2) were given a restricted age range of 30.7–40.3% (95% confidence intervals in Figure 4g). The gap between feeding and postfeeding third instars closed somewhat in more complex models; model 18, which utilized all available data, showed no gap between these stages (Figure 4h), although the data still did not cluster along the Y = X line at the level seen during feeding. The inaccuracy of predictions remained for pupae in all models, where true pupal ages were between 43.2–100% of immature development, but 95% of predictions for pupae using model 18 had fitted values between 61.9–81.2%. Interestingly, predicted ages throughout this range were made for pupae of any true age; that is, there was no slope to the pupal data as there was for the other stages.

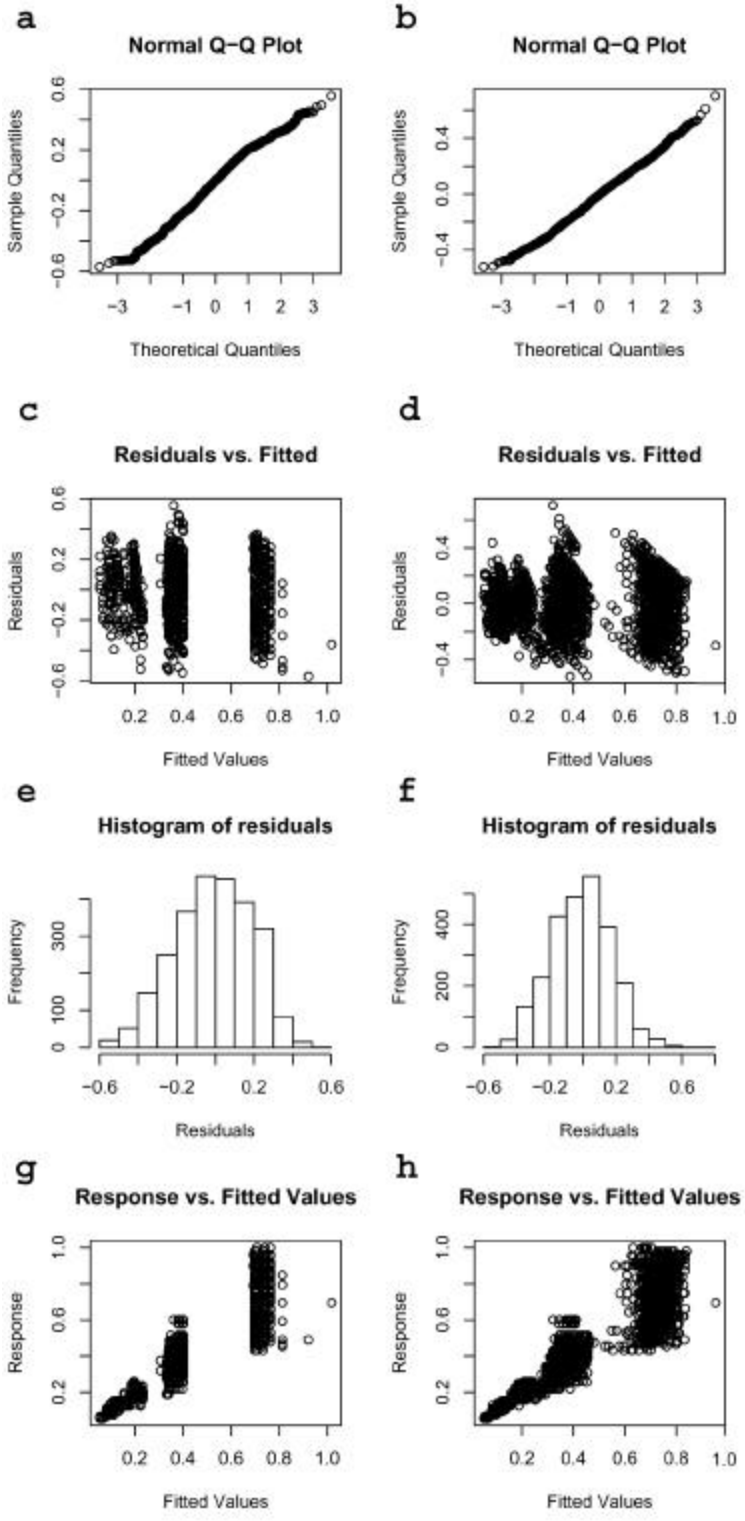

Figure 4. A comparison of diagnostic plots for model 10 (a, c, e, g) and model 18 (b, d, f, h). Model 10 predicted age using length and stage, while model 18 used all data to make age predictions, and explained the most deviance in the data. Panels a) and b) are quantile-quantile plots, assessing the validity of each model's assumptions. The line is relatively straight and

increasing for both models, indicating that the distributional assumption of the model did not violate the other assumptions of the model, however model 18 generated a smoother line. Panels c) and d) show the distribution of residuals throughout the lifecycle; in both models residuals are of equivalent size for all ages, showing that there is no bias in residuals based on age. Panels e) and f) are the distributions of residuals, which are normally distributed thus the gamma distribution was acceptable to use with these data. Panels g) and h) are plots of true (response) versus predicted (fitted) values for data used to construct the models. Fitted values accurately predicted true age through linear (feeding) development (the first 25% of the lifecycle), after which the first gap in prediction appeared. This indicates that an overestimation of ages for postfeeding larvae between *c*. 19.1 and 30% developed was produced using model 10. Model 18 was less likely to overestimate age for individuals at this point in the lifecycle, but predictions were still biased toward overestimating age in young postfeeding third instars. Neither model represents a noticeable improvement over using stage alone for aging pupae. Predicting pupal age with just developmental stage resulted in an age estimate between 43.2 and 100% of immature development. The most predictive model (model 18) plots fitted values between 61.9 and 81.2 % (pupae). At either end of this predicted range, true values could be between 43.2 and 100%. As with all size/stage models, error increased with age.

GAM Validation with Independent Data.

The utility of model 10 (developmental stage and length) was examined through analysis of the previously collected and independently produced rat carcass data set. Consistent with the finding above, error in larval age estimations increased with age (pupae were not considered here as length does not change during the stage). A plot of true versus predicted age (Figure 5) shows that age predictions generated for the rat data, when compared to known ages, spanned the Y = X line and were generally consistent with (inside) the 95% confidence interval provided by the diagnostic plot for model 10. In the feeding stages (<26% of total development) the predictions were approximately +/-5% (or less) of the true age. However, in postfeeding third instars, ages were initially overestimated, then clustered close to the line, and eventually disbursed well below Y = X, consistent with the expectation that postfeeding individuals could not be precisely aged using length. The model also continued to predict a narrower range of ages for postfeeding larvae (32.5%–40.1%) as compared to their true ages (22.9%–50.2%).

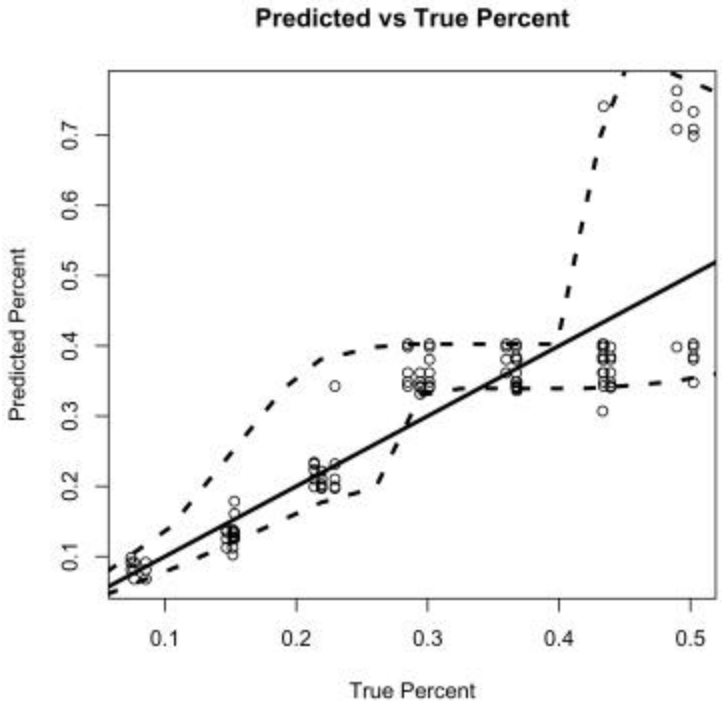

Figure 5. A plot of predicted versus true ages of 252 individual flies raised on rats as estimated with a GAM for stage and length (model 10). A plot of predicted versus true development percents of 252 larvae raised on rats as estimated with a GAM for developmental stage and length (model 10). Development is displayed to 50% because length measurements ceased when pupation occurred. The solid line represents the Y (predicted age) = X (true age) line. Dashed lines represent 95% confidence intervals for the predictions based on the data in (a). Model 10 accurately (data span Y = X) and precisely (ranging c. 5% above and below Y = X) predicted age for the feeding portion of the lifecycle (<26% of development), when body size increased in a linear fashion. As expected, precision decreased greatly once the postfeeding third instar was reached, although overall error for the model was within predicted levels.

Discussion

The requirements of Daubert necessitate standardized methodologies and knowledge of potential error, two criteria where several forensic sciences, including entomology, may be found lacking. Previously we examined how variation in published rearing protocols, which are not standardized among laboratories, affect growth rates of juvenile blow flies (Tarone and Foran

2006). In the current research the ability to conduct statistical analysis of blow fly growth and aging, including confidence intervals, error rates, and model comparisons, was tested. From a practical standpoint, the methodology allows for direct estimates of error that should satisfy both scientific and Daubert considerations. For instance, if stage and length are used to estimate that a larva is 15% developed, model 10 generates a 95% confidence interval of approximately 10–22% (Figure 5). Using a published minimum development time for *L. sericata* of 288 h at 26.7ºC (Kamal 1958), an age estimate of 29–52 h is produced, with the requisite error described. For postfeeding third instars, an estimate of 40% developed (115 h) corresponds to a 95% confidence interval of approximately 22– 60%, or 63–173 h. Though the precision necessarily decreases in the latter stages, the window of time placed around that prediction is now mathematically defined. The methodology also has the flexibility to incorporate other data that may be collected.

Through the modeling used in this study, several key points became apparent. First, developmental stage was the single most predictive factor in the models assessed, explaining 89.5% of the deviance in the data. Logically this makes sense, as stage is a direct measurement of developmental progress. In contrast, the non-linear measurements—weight and length—although still significant, proved far less effective in predicting development, while strain and temperature (genetic and environmental factors) were by themselves not significant predictors. The ability of stage, length, and weight to estimate age was greatest during the earliest phases of development, but for different reasons. Egg, first instar, and second instar are by far the shortest developmental stages in flies (Figure 2), thus identification of any of these simply described development more accurately than did the much longer third instar and pupation. Weight and length on the other hand are related to feeding, and changed in a relatively linear fashion during

the early larval stages, including the first portion of the third instar (e.g., Figure 1), but once feeding ceased their utility dropped dramatically due to the reversal in body size and larger overall variance in length and weight. Likewise, pupal size was of little utility as it is static throughout the stage. As a result, model 18, which used all available information, predicted a restricted pupal development of 61.9% to 81.2% (Figure 4h, 95% confidence intervals) and showed no specificity (that is, the youngest or oldest individuals were equally likely to be placed anywhere within that range). This means a pupal development prediction with the best GAM was essentially the same as using stage alone. Given these effects on predictive ability, it is not surprising that adding weight and/or length to stage resulted in minimally improved models.

Second, error increased as development progressed for all models, indicated by the gaps in predictive ability and the widening confidence intervals for successive developmental stages (Figure 4g and h), which were most pronounced in postfeeding third instars and pupae. The increasing inaccuracy of age approximation as fly development progresses has been noted in the literature (Wells and Kurahashi 1994, Wells and Lamotte 1995), and forensic entomologists account for it in PMI estimates by giving large age ranges to postfeeding flies, although these rarely include an objective estimate of error. The latter study (Wells and Lamotte 1995) used linear models to estimate blow fly age based on length data, and yielded an increase in error for older larvae. The similar findings indicate that there is a limit to the precision in blow fly age predictions that can be achieved when only developmental stage and body size are evaluated. Owing to this, alternative developmental data independent of basic growth are likely required to increase the accuracy of PMI predictions, and in the future, traits that change regularly during fly development, such as hormone titers or gene expression, may be useful in generating a more specific PMI.

Third, the limited influence of fly strain and rearing temperature on development is an important consideration as it indicates the models have value regardless of where flies are collected or at what temperature they develop. This is not to say that temperature is unimportant when making PMI estimates—it is critical, and is always considered when estimating PMI (usually as accumulated degree days). However, temperature did not alter developmental patterns to any large extent, although lower rearing temperatures did result in slightly larger individuals overall for all strains, a finding we continue to investigate. Similarly, the strains of flies examined had different average sizes during development (Figure 3). For both criteria the distributions of body size throughout development overlapped, so these data modified age predictions minimally. Also, there was little difference in size among strains during the feeding stages of the lifecycle, where size best predicts age, thus size variability resulting from strain adds no confounding information during those stages.

Fourth, any given forensic case may present the entomologist with different data from which to estimate fly age. While developmental stage was the most useful datum for the development estimates in this study, other data, such as weight and length, increased their accuracy. Using a model that incorporates all available data can help ensure that investigators make the best possible prediction with the information they receive, while maintaining an understanding of the limitations inherent in that model. An estimate of the relative reliability of a PMI prediction (based on the GCV and percent deviance explained) provides an understanding of its value in interpreting evidence. Overall, generalized additive models offer a useful means of incorporating information from multiple linear and non-linear variables to predict blow fly age, variables that can be accommodated even if they change from one case to the next.

Finally and most importantly, a comparison of modeled development predictions to the independently derived rat data made it possible to assess error rates and produce confidence intervals in these estimates. Individuals less than 26% developed (feeding larvae) generated the most accurate predictions; when 12 individuals of the same age were sampled from a cohort, the predictions clustered around the known age in all instances (Figure 5). In contrast, postfeeding stages had a much larger error rate. It is worth noting that even when model 10 yielded its best estimates, there was still about 10% total variance in predicted ages of larvae (note again, at a true age of 15%, the 95% confidence interval for rat data predictions was between *c*. 10–22%, thus stage and size were not 'perfect' in estimating development even in the youngest individuals. The utility of the methodology presented here is that it establishes a defined way to produce confidence intervals around entomologically based PMI predictions, regardless of fly age. Until new and independent variables that change in a predictable manner during development are incorporated into age estimates, this error will necessarily exist, and increase with age, however through these models that error can objectively be determined. Equipped with such knowledge the forensic entomologist can relay to the court the level of error found in a PMI prediction. Through this feat, one of the major requirements of Daubert is more fully addressed.

TABLE 1—The 18 generalized additive models for predicting development percent assessed in this experiment.

Model	Development Percent=	Percent	GCV
1	Stage	89.5	0.045
2	s(Length)	63.3	0.126
3	s(Weight)	65.7	0.144
4	s(Length,Weight)	86.8	0.059
5	Temperature	0.022	0.358
6	Strain	0.0041	0.358
7	Stage+Strain	89.5	0.044
8	Stage+Temperature	89.7	0.044
9	Stage+Strain+Temperature	89.7	0.044
10	Stage+s(Length)	91.2	0.038
11	Stage+s(Weight)	90.8	0.04
12	s(Length)+s(Weight)	85.9	0.064
13	Stage+s(Length)+s(Weight)	91.6	0.036
14	Stage+s(Length)+s(Weight)+s(Length,Weight)	92	0.035
15	Stage+s(Length)+s(Weight)+Temperature	91.8	0.036
16	Stage+s(Length)+s(Weight)+Strain	91.6	0.036
17	Stage+s(Length)+s(Weight)+Temperature+Strain	92	0.036
18	Stage+s(Length)+s(Weight)+s(Length,Weight)+Temperature+Strain	92.6	0.034

s(variable)- Indicates that a smoothing curve was used in the GAM for this variable.
s(Length,Weight)- Indicates that a smoothed contour surface of length plotted against weight was used in the GAM.
Development percent- Indicates the variables used in each model to predict development percent.
Percent- Indicates the percent deviance explained.
GCV- Generalized cross validation score; lower scores are better models for predicting development percent.

TABLE 2—Summaries of the estimated significance of each term in each model.

Model	Variable	df or edf	Chi-sq	P-value
1	Stage	4	26824	<0.0001
2	Length	8.858	7148.5	<0.0001
3	Weight	7.435	6749.8	<0.0001
4	Length,Weight	27.45	19814	<0.0001
5	Temperature	1	0.0685	0.41
6	Strain	2	0.129	0.94
7	Stage	4	26917	<0.0001
	Strain	2	6.4603	0.04
8	Stage	4	27120	<0.0001
	Temperature	1	52.474	<0.0001
9	Stage	4	27201	<0.0001
	Strain	2	5.1408	0.077
	Temperature	1	51.348	<0.0001
10	Stage	4	10933	<0.0001
	Length	8.761	529.96	<0.0001
11	Stage	4	7939.8	<0.0001
	Weight	7.902	377.69	<0.0001
12	Length	8.333	3959.5	<0.0001
	Weight	8.124	5395.6	<0.0001
13	Stage	4	1951.2	<0.0001
	Length	8.63	275.02	<0.0001
	Weight	5.186	107.81	<0.0001
14	Stage	4	1476.5	<0.0001
	Length	8.864	73.484	<0.0001
	Weight	7.139	19.551	0.0074
	Length,Weight	21.08	118.99	<0.0001
15	Stage	4	1913.1	<0.0001
	Length	8.598	273.65	<0.0001
	Weight	5.83	112.08	<0.0001
	Temperature	1	85.259	<0.0001
16	Stage	4	1884.8	<0.0001
	Length	8.648	282.94	<0.0001
	Weight	6.139	126.42	<0.0001
	Strain	2	26.24	<0.0001
17	Stage	4	1845	<0.0001
	Length	8.654	295.57	<0.0001
	Weight	7.473	140.54	<0.0001

	Temperature	1	105.07	<0.0001
	Strain	2	45.732	<0.0001
18	Stage	4	1409.6	<0.0001
	Length	8.9	70.164	<0.0001
	Weight	4.187	38.203	<0.0001
	Length,Weight	27	212.6	<0.0001
	Temperature	1	156.6	<0.0001
	Strain	2	100.68	<0.0001

df- Degree of freedom.
edf- Estimated degree of freedom (non-parametric variables).
Chi-sq- Chi-squared score.
P-value- As estimated from likelihood scores for each parameter in a model.
Figure 1

Gene Expression in Fly Eggs (The following section is under review in the Journal of Forensic Sciences)

Insects found on human remains can be useful in estimating a postmortem interval (PMI) during death investigations (Catts and Haskell 1990). Primary among these are the blow flies (Diptera: Calliphoridae), whose state of development when collected from a corpse can be compared to published tables of juvenile fly growth, in order to approximate when the eggs were deposited. As development continues, the larvae pass through three instars and then move away from their food source in order to pupate. For many necrophagous fly species, including the widely distributed blow fly *Lucilia sericata*, growth rates are well defined (e.g., Kamal 1958, Greenberg 1990, Anderson 2000, Grassberger and Reiter 2001). However, developmental stages necessarily exist over a period of time, in some cases several days, making precise PMI estimates difficult. Given this, any information that could be added to fly development stage data has the potential to generate a more precise PMI.

While outward characteristics such as body size or instar have generally been used to estimate fly age, other traits that are developmentally regulated, including the differential expression of genes, offer great potential as an independent source of data for estimating blow fly age. Developmental biology research has uncovered numerous instances of gene expression changes throughout maturation (see Kalthoff 2001, and references therein). Flies have been particularly well studied in this regard (Henrich and Brown 1995, Arbeitman et al. 2002, Skaer et al. 2002, Sullivan and Thummel 2003, Luders et al. 2003, Ali et al. 2005, McGregor 2005), including the Calliphoridae. Predictable changes in gene expression during development led to the hypothesis tested here, that differential gene expression could be used to make more precise

PMI predictions, by effectively breaking a developmental stage into smaller developmental units. Towards this goal, mRNA levels of three genes differentially expressed in *Drosophila melanogaster* eggs (Arbeitman et al. 2002), *bicoid* (*bcd*), *slalom* (*sll*), and *chitin synthase* (*cs*), were assayed in *L. sericata.* Eggs were chosen because there is no quantitative means of assessing their degree of maturity, and if egg aging is attempted at all, investigators must rely on a qualitative evaluation of embryos, making it difficult to objectively divide the stage into developmental subgroups. *bcd* is required early in egg development, defining the anterior end of the egg during the formation of the anterior-posterior axis in Cyclorrhaphan flies (McGregor 2005), including the Drosophilidae and Calliphoridae. *sll* affects dorsal-ventral patterning (Luders et al. 2003), and is also highly expressed in the salivary glands of *D. melanogaster* and *L. sericata* larvae (Ali et al. 2005). *cs* was profiled as chitin formation is required for the proper assembly of the larval cuticle (Tellam et al. 2000). Transcript abundances were assessed to directly test the hypothesis that developmental stages of forensically important flies can be better defined by combining expression information from specific genes, resulting in more precise age estimates, as well as a more precise prediction of PMI.

Materials and Methods

Species Identification and Egg Collection

L. sericata was collected in East Lansing, Michigan, and was identified visually and genetically as previously described (Tarone and Foran 2006). A fly cage at room temperature was presented with beef liver and examined every 15 minutes until females were observed laying eggs, which was allowed to continue for one h. Egg masses (comprised of approximately 250 eggs) were placed on a moist paper towel in a petri dish at 32ºC, and whole masses were

collected hourly until hatching of the remaining eggs was observed. Sampled masses were immediately fixed in RNA Later (Applied Biosystems, Foster City, CA) and stored at -80ºC. Two replicates were collected for each hourly age period. Prior to RNA extraction, egg masses were thawed and sliced with a razor blade into fifths, resulting in the analysis of 10 groups of eggs for each one-hour collection span. The first eggs hatched between 9 and 10 h, thus the 8–9 h time span was the oldest analyzed.

Gene Sequencing and Primer Design.

Expression levels of *bcd*, *sll*, and *cs* were compared to the steady-state expression of two housekeeping genes (*rp49* and *ß tubulin 56D*). *L. sericata* gene sequences were available for *bcd*, *sll*, and *rp49* on the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov), thus quantitative PCR primers were designed directly from them using Applied Biosystems Primer Express software. *ß tubulin 56D* and *cs* sequences were obtained using primers for the *D. melanogaster* and *L. cuprina* genes respectively (Table 1), taken from NCBI. PCR consisted of 35 cycles of denaturing at 95ºC for 30 seconds, annealing primers at 55ºC for 30 seconds, and extending amplicons at 72ºC. Extension times were 4 min for *cs* and 2 min for *ß tubulin 56D*. Sequences were generated on a CEQ 8000 capillary electrophoresis system using a CEQ DTCS Quick Start Kit and the same primers, following the manufacturer's protocols (Beckman Coulter, Fullerton, CA). PCR products were analyzed via agarose gel electrophoresis; a single peak in dissociation curves of quantitative PCR (see below) confirmed the electrophoretic evaluation.

cDNA Synthesis and Quantitative PCR.

Ninety RNA samples were isolated from egg masses in a 96-well format using an ABI PRISM 6100 Nucleic Acid PrepStation and the manufacturer's solutions and protocols (Applied

Biosystems). Eggs were placed in 300µL of lysis solution without use of a pre-filtration plate. RNA was eluted from plates with 100µL elution solution and incubated at 37ºC for 1 h with 70 units of DNase-I (RNase-Free, Roche, Basel, Switzerland) and Ambion DNaseI buffer (Applied Biosystems). The enzyme was inactivated at 75ºC for 10 minutes and the RNA was precipitated using 110µL of isopropanol followed by centrifugation at maximum speed for ½ h at 4ºC. Two 70% ethanol washes followed, using the same centrifuge settings. RNA samples were allowed to air dry for 15 minutes, at which point 32µL Ambion RNase-Free water (Applied Biosystems) was added to the pellet prior to freezing at -80ºC.

cDNA was synthesized using a TaqMan Reverse Transcriptase kit (Applied Biosystems) primed by oligo(dT) 16-mers according to the manufacturer's instructions, including 30µL of RNA in a final volume of 120µL. Gene expression levels were assessed by quantitative PCR on an Applied Biosystems 7900HT using SYBRgreen PCR mastermix (Applied Biosystems) in 15µL reactions on a 384-well plate. Each reaction received 1.5 µL cDNA, 7.5 µL SYBRgreen PCR mastermix, and 1 µL each of forward and reverse primers. The Applied Biosystems recommended thermal cycling parameters were used with the exception that PCR cycles were increased to 50 and a dissociation curve was produced for every reaction. Results were considered valid if a single peak was present in the dissociation curve, indicative of a single amplicon being produced. Optimized primer concentrations, based on trial runs designed to ascertain concentration combinations that provided the largest signal to noise ratio in dissociation curves, are found in Table 4.1.

Reactions without reverse transcriptase acted as controls to confirm that amplification in quantitative PCR was not due to residual DNA. A known (positive) cDNA sample was analyzed in triplicate during every run, allowing for comparisons among 96-well plates, resulting in ninety

cDNA samples (10 per time point, in duplicate), six negative controls (PCR mix with no DNA), and six positive controls being assessed for each locus.

Statistical analyses and the construction of plots were performed in the R statistical program (R core development team 2004). Linear regression models were analyzed via type III ANOVA. Standardized gene expression through time was plotted for samples that yielded detectable levels of a transcript. The use of gene expression to assess age was examined via generalized additive models (Hastie and Tibshirani 1990, Wood 2006), which produce a statistic, percent deviance explained (similar to R^2), assessing the extent to which a variable influences the data. Predictions (fitted values) for the data were plotted against true ages (response), allowing for evaluation of the model's ability to predict the egg ages.

Final CT values for all loci were generated using the average of duplicate PCRs. CT values of *rp49* and *ß tubulin 56D* were averaged and subtracted from those of the developmentally regulated genes to obtain a standardized CT. Regression curves were drawn through standardized plots. Binary gene expression values (1 = present, 0 = not present) were also assessed to determine if the presence or absence of gene expression corresponded to a particular age. A locally weighted sum of squares curve was drawn through the resultant plot. Generalized additive models then allowed prediction of egg age with CT scores and binary values. One model used binary *cs* expression and CT information from the other loci to predict age (N = 55). The other estimated age with CT data for all loci (N = 33). Sample sizes were smaller than the total as only egg masses that provided data for all loci were included in analyses.

Results

L. sericata sequences for *ß tubulin 56D* and *cs* are listed under the National Center for Biotechnology Information accession numbers EF056211 and EF056212 respectively. *cs* best matched its *L. cuprina* homolog (98% with no gaps) and *ß tubulin 56D* exhibited the closest similarity to the fly *Glossina morsitans morsitans tubulin beta-1* gene (86% identity with no gaps); no *Lucilia* sequence was available for the latter comparison.

Eighty-four of the 90 samples yielded *rp49* and *ß tubulin 56D* profiles, which demonstrated consistent expression levels throughout egg development. There was a significant positive relationship in expression of the two housekeeping genes ($P<0.0001$, $R^2=0.63$), confirming their utility as internal standards. Of the 84 samples, *bcd*, *cs*, and *sll* had an undetectable transcript level in 23, 31, and 20 samples respectively.

The developmentally regulated genes demonstrated qualitative and quantitative differences in expression throughout egg development. *cs* was the only gene that showed a consistent binary (on/off) pattern, with egg masses less than two h old never producing the transcript, while those 6 h and older always expressed the gene, therefore *cs* expression state could be plotted during egg development (Figure 1). The presence of the transcript was a statistically significant predictor of age ($P<0.0001$, $R^2=0.59$).

Each of the genes had a different quantitative expression pattern (Figures. 2–4; note that the displayed CT values are inversely related to gene expression levels). Though only expressed after hour 2, *cs* transcripts significantly increased during egg development ($P=0.0004$, $R^2=0.21$). Conversely, *bcd* and *sll* transcripts were at their highest levels and lowest variation at 0–2 h, and significantly decreased as development proceeded ($P=0.0003$, $R^2=0.19$ and $P=0.0023$, $R^2=0.13$ respectively).

Finally, generalized additive models were used to predict egg ages based on the gene expression data. The first model used the binary expression data for *cs* and CT scores for *bcd* and *sll* to predict egg age. It explained 72.1% of the deviance in the data and accurately enabled the identification of egg masses as either 0–4 or 2–9 h old (not shown). Next, CT scores for all three genes were used to predict age, which explained 76.7% of the deviance in the data. When predicted versus true ages were plotted (Figure 5), estimated ages followed the True = Predicted line, with 30 of 33 predictions within 2 h of the true age.

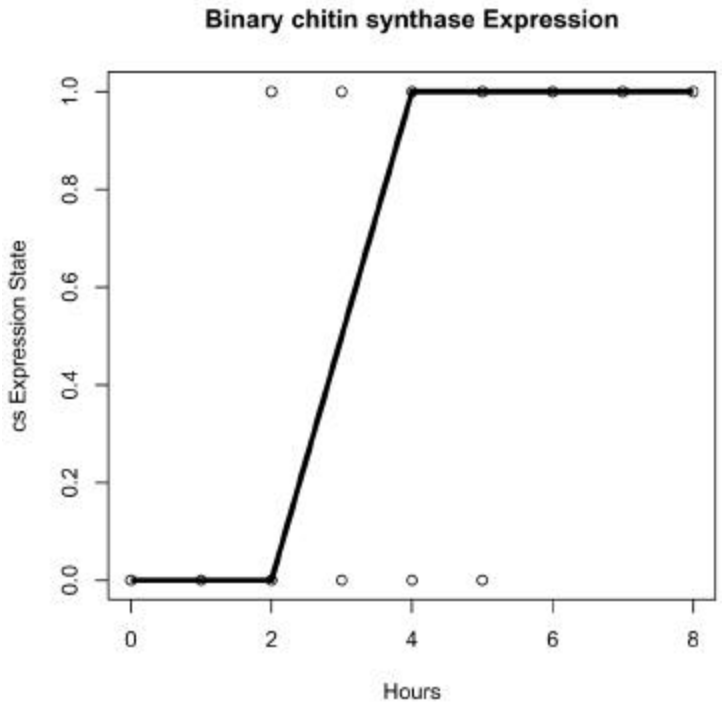

Figure 1. Binary gene expression profile for *cs* at 32ºC in *L. sericata* eggs, from 0–1 through 8–9 hours of development. 0 indicates no detectable expression of the gene and 1 indicates detectable expression of *cs*. Expression was not detected from 0–2 hours. Between 2 and 6 hours some eggs clusters expressed *cs* and some did not. After 6 hours, all egg clusters expressed *cs*.

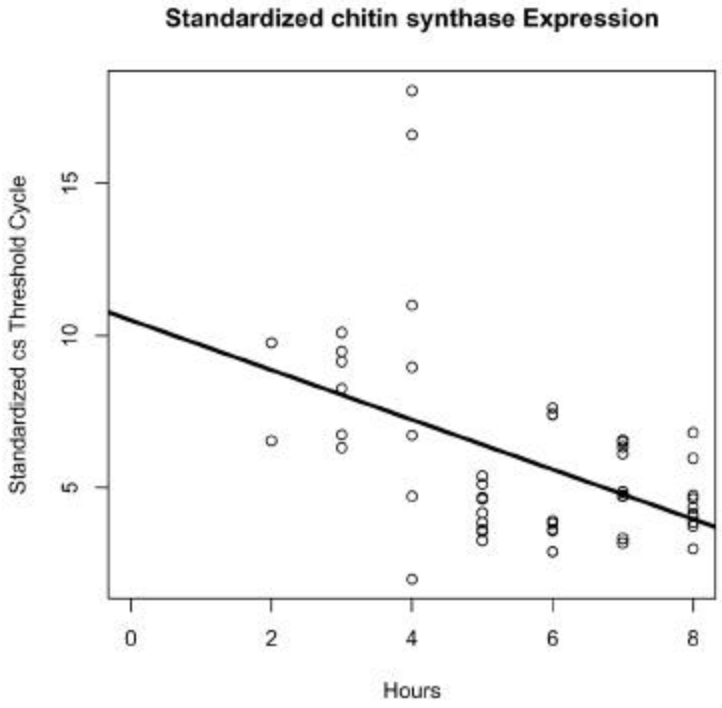

Figure 2. Standardized expression of *cs* in *L. sericata* eggs, from 0–1 through 8–9 hours of development. The CT was standardized against the average of *rp49* and *ß tubulin 56D* CT values and plotted through time. The regression of *cs* CT over time was also included. High expression levels are indicated by low CT values. *cs* was not expressed from 0–2 hours and then its expression increased.

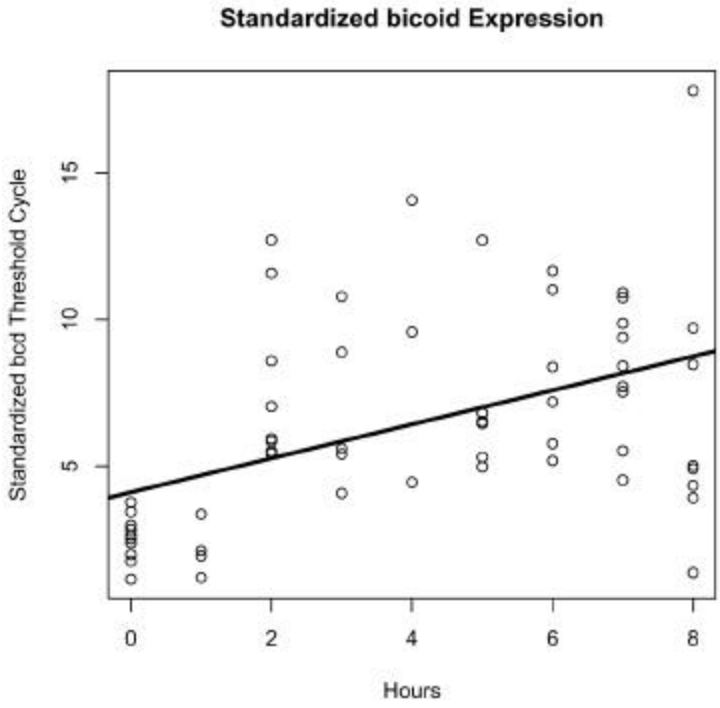

Figure 3. Standardized transcript abundance of *bcd* in *L. sericata* eggs, from 0–1 through 8–9 hours of development. Standardization was as in Figure 4.2. *bcd* gene expression was highest from 0–2 hours, then transcripts decreased in abundance.

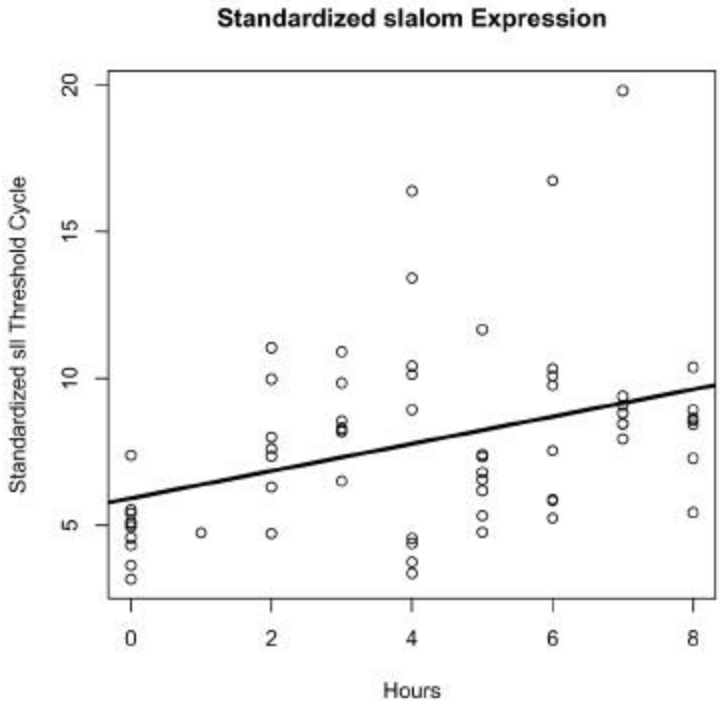

Figure 4. Standardized transcript abundance of *sll* in *L. sericata* eggs, from 0–1 through 8–9 hours of development. Standardization was as in Figure 4.2. *sll* expression was highest from 0–2 hours, then tended to be lower as eggs developed, though variance in expression was high.

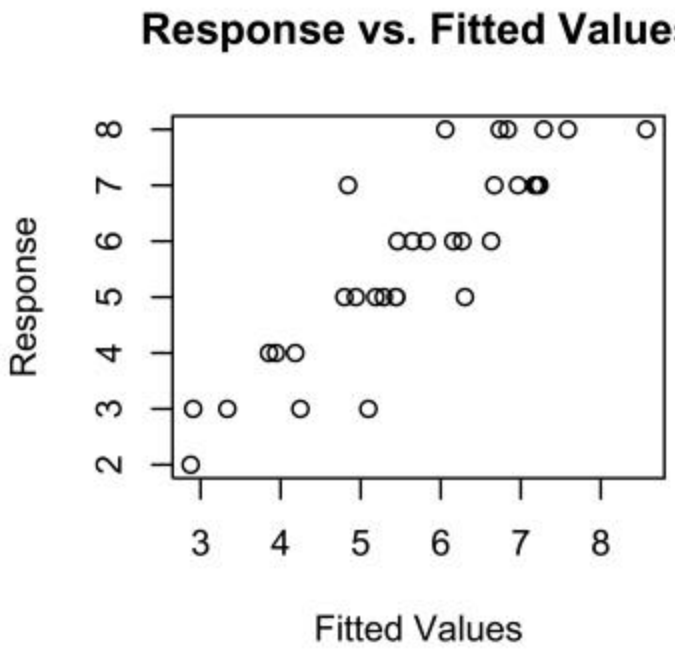

Figure 5. Predicted (fitted) versus true (response) ages for a generalized additive model that made age predictions for the 33 egg masses that expressed *bcd*, *sll*, and *cs*. Estimated ages were within 2 h of the true age in 30 of the 33 cases.

Discussion

The goal of this research was to examine the feasibility of using gene expression to more precisely age immature flies of forensic interest, consequently generating more accurate estimates of PMI. The loci examined demonstrated statistically significant, though noisy, trends in expression levels throughout egg development. Additionally, egg masses less than 2 h old did not express *cs*, while egg masses older than 6 h always expressed the gene. Following on efforts to predict adult mosquito age using gene expression and multiple regression (Cook et al. 2006), generalized additive models were used to predict egg ages. When CT scores were available for all loci, 91% of predictions were within 2 h of the true age, while the binary *cs* data combined with *bcd* and *sll* CT scores separated the egg masses into two distinct groups.

A key factor in aging flies using gene expression is, of course, examining loci likely to vary in expression levels during the developmental period being examined. In eggs, genes that are important for developmental patterning (e.g., dorsal/ventral) are crucial for successful growth of the individual, thus their expression is under strict biological control. During very early fly embryogenesis high levels of maternally derived *bcd* and *sll* products are necessary to properly establish biological axes (Kalthoff 2001, Arbeitman 2002, Luders et al. 2003, McGregor 2005). In the current study, transcript levels of both genes dropped steadily through embryonic development, although neither became undetectable. In *D. melanogaster*, *bcd* is detectable only during early embryonic development (Arbeitman et al. 2002), thus its expression throughout embryogenesis in *Lucilia*, albeit at decreasing levels, is somewhat puzzling. Developmental heterogeneity among eggs may have resulted in this phenomenon, while it is also possible that *bcd* serves some unknown secondary function in blow flies, or that the transcript is stable but untranslated in older eggs. In contrast, the existence of *sll* transcripts at the end of the egg stage can be accounted for, likely resulting from endogenous *sll* expression commencing in the developing salivary glands (Luders et al. 2003). Finally, *cs*, which is required only for production of the larval cuticle (Tellam et al. 2000), followed a different and predicted transcriptional course, wherein the earliest portion of egg development was defined by an absence of transcripts, the middle portion of development, as larval cuticle begins to form, was represented by low to intermediate levels of *cs* expression, and the highest levels were found late in egg development. Most importantly, all three loci showed significant trends in egg transcript expression over time, and taken together increased the precision of egg age estimates.

Given that gene expression has the potential to more accurately age flies of forensic interest, other factors need to be considered, including both the feasibility, and legal acceptance,

of the methods. The molecular techniques employed in this study have been widely utilized in developmental molecular biology, and as important, could readily be implemented in most laboratories equipped for forensic DNA investigation. They also provide information that can be used to generate predictable error rates/confidence intervals, meeting one of the major tenets of Daubert, an important consideration of any new forensic protocol. The methods are amenable to microarrays (e.g. Arbeitman et al. 2002) and robotics, potentially producing simplified and high throughput blow fly aging analyses. Finally, DNA-based methodologies overall have been widely accepted in courts of law, thus new but related methods should have less difficulty overcoming Daubert challenges.

The data presented here demonstrate that even the briefest phase of fly development, the egg stage that lasts only several hours, can be divided into smaller periods using gene expression data. Naturally, other stages of fly development, particularly those that last longer and therefore are more forensically challenging (e.g., the third instar and pupation) can be examined using these methods as well. Addition of more developmentally regulated genes into the analysis should further increase the precision of age estimates by providing more age-informative data. The final outcome of this is a more precise age given to developing blow flies, resulting in more precise estimates of PMI.

TABLE 1. Primers used in this study.

Primer	Function	Sequence	Accession Number	Concentration (nM)
qBcd F	Quantitative PCR	CCTCGCTCCGGGCCCATAAC	AJ297856	400
qBcd R	Quantitative PCR	CAACTACTGGCACTTCCATTTGAA	AJ297856	400
qTub F	Quantitative PCR	TCCGTAAATTGGCCGTCAAC	EF056211	400
qTub R	Quantitative PCR	ACCAGGCATGAAAAAGTGAAGAC	EF056211	400
qCs F	Quantitative PCR	GCCGACGGAGAACCTATACCA	EF056212	66.67
qCs R	Quantitative PCR	GATGGCTGTCATTGTGGGTACA	EF056212	400
qSll F	Quantitative PCR	TCCAACGGCCACAATCTTAAGTA	AY926574	66.67
qSll R	Quantitative PCR	CGTTTAGGTGTTGCCGCAAT	AY926574	400
qRp49 F	Quantitative PCR	ACAATGTTAAGGAACTCGAAGTTTTG	AB118976	400
qRp49 R	Quantitative PCR	GGAGACACCGTGAGCGATTT	AB118976	400
Tubulin F1	Sequencing	CGAGACCTACTGCATCGACA	NM_166357	1000
Tubulin R1	Sequencing	CACCAGATCGTTCATGTTGC	NM_166357	1000
Cs F2	Sequencing	GAACTGCCTATACCCGTGGA	AF221067	1000
Cs R2	Sequencing	GGATGTAAACACGCCGCTAT	AF221067	1000

Accession number denotes the sequence from which the primers were derived. Concentration denotes the final concentration (nM) of primers used in each PCR reaction.

Larval and Pupal Gene Expression

The main hypothesis of this research was that the use of gene expression will increase the precision of PMI predictions derived from entomological evidence. The premise was based on two basic observations. First, later stages of immature fly development have longer durations and more variation in body size (Kamal 1958, Greenberg 1990, Wells and Lamotte 1995, Anderson 2000, Grassberger and Reiter 2001, Greenberg and Kunich 2002, Tarone and Foran 2006), necessitating larger windows around age predictions for those stages, thus less precise estimates of age. The section on Generalized Additive Models demonstrates this point keenly, indicating a need for new data to make precise predictions. Additionally, the wealth of developmental genetic data for *D. melanogaster* and other fly species (Kalthoff 2001, Arbeitman et al. 2002, Skaer et al. 2002, Beckstead et al. 2005) indicates that gene expression levels vary reliably throughout the immature stages that are of interest to forensic entomologists, and are a unique form a data that might be incorporated into the PMI prediction process. The goal of this project was to identify and use developmentally variable gene expression patterns to predict blow fly age in the widespread and forensically informative species *L. sericata.* In this section gene expression profiles for nine developmentally variable genes and two housekeeping genes were produced for a 958 individual subset of the collections from the Generalized Additive Models section. The data produced were then used to construct and compare GAMs (Hastie and Tibshirani 1990, Wood 2006) examining minimum development percent. Results were positive, with GAMs utilizing gene expression data producing more precise predictions than models that did not predict age with gene expression.

Materials and Methods

Species Identification, Fly Rearing, and Collections.

L. sericata rearing methods and species confirmation are detailed in the Generalized Additive Models section above. Each collected individual was immediately frozen in RNAlater (Applied Biosystems), at –80ºC after length, weight, and developmental stage were recorded.

Sequencing of *L. sericata* Loci.

Several loci required sequencing before quantitative PCR primers could be designed. These included *ß Tubulin 56 D, chitin synthase* (both detailed in the Gene Expression in Fly Eggs section above), *acetylcholine esterase, ecdysone receptor, ultraspiracle, white, scalloped wings,* and *rhodopsin 3*. Sequences from the closest dipteran relative available at (www.ncbi.nlm.nih.gov) were used to design PCR primers for the locus, targeting at least a 300 bp segment. Sequencing primers and the sequences used to design them are listed in Table 1. Sequencing methods were as in Gene Expression in Fly Eggs, with slight modifications of annealing temperature, extension times, and the number of cycles depending on the gene in question.

RNA Extraction.

RNA was isolated from a subset of the 2559 individuals described in the Generalized Additive Models section. Five individuals per time point were examined from the Michigan and California replicates raised at 20ºC or 33.5ºC, and from the West Virginia replicates raised at 33.5ºC. The RNA isolation method from Gene Expression in Fly Eggs, conducted in a 96-well format on an ABI PRISM 6100, was modified for use with larvae and pupae, stemming from the fact that adding too much lysed tissue to the wells prevented solutions from being drawn through the filters. Flies were ground in 300 µL of lysis solution by hand with a sterile pestle. Lysates

from larvae greater than 10 mg in size and from pupae were diluted in RNA lysis solution (Applied Biosystems); twenty µL of larval lysates or 40 µL of pupal lysates, were placed into 300 µL of additional RNA lysis solution. The dilutions were determined in preliminary experiments to establish the largest volume of preliminary lysate that did not clog the filters of the 96-well RNA isolation plates. The diluted 300 µL lysates of individual larvae and pupae of any size were then drawn through a 96-well filter plate (Applied Biosystems), which removed large particles, larval cuticle, and pieces of puparium from the solution before it was added to the RNA isolation plate. All other steps were followed using the manufacturer suggested protocol, with a final RNA elution volume of 100 µL. After RNA purification a DNase I reaction removed any remaining DNA contamination as described in Gene Expression in Fly Eggs.

Reverse Transcription and Quantitative PCR.

cDNAs and controls were developed as in the Gene Expression in Fly Eggs section, except that a High Capacity cDNA Archive Kit (Applied Biosystems) was utilized , according to the manufacturer's instructions. Once the no reverse transcriptase controls were shown to be negative (DNA free) using the *rp49* primers, quantitative PCR was performed with primers for all genes (loci, qPCR primers, and primer concentrations are in Table 1). All qPCR products yielded one product of the appropriate size when checked by gel electrophoresis, and dissociation curves were consistent with a single product. Any reactions that had deviant dissociation curves were eliminated from the study.

Quantitative PCR was performed using the same cycling parameters as in Gene Expression in Fly Eggs, however quantitative PCR was set up and analyzed in a 384-well format using a Biomek 2000 Automated Workstation (Beckman Coulter), not by hand. qPCR reactions were performed with 10 µL reactions, which consisted of 2 µL of cDNA. Power SYBR Green

PCR Master Mix was used in the reactions, instead of the SYBR Green PCR Master Mix (Applied Biosystems) used in Gene Expression in Fly Eggs.

Samples of cDNA from an individual were divided into two wells and the average CT was used as the score for that gene and individual. Scores were standardized against an internal standard value derived from the average of the housekeeping gene CTs (*rp49* and *ß tubulin 56D*) by subtracting the housekeeping score from the gene CT. In addition, reactions were standardized against each other by setting the CT for the positive control reactions to the same value on every 384-well plate in the experiment (positive control reactions were run with *rp49* primers and a standard cDNA sample).

Statistical Analyses.

Statistics and graphs were produced using the R statistical package (R core development team). Several plots were used to understand the data. Gene expression was compared for each developmental stage using boxplots of standardized gene expression CT scores for each locus. Descriptions of boxplots can be found in the Generalized Additive Models section. Standard CT values for each locus were plotted against minimum development percents as detailed in Generalized Additive Models, and locally weighted sum of squares curves were drawn through the non-linear data, allowing comparisons of average expression to temperature treatments and average expression among strains. For the graphs (see Results), a gene for which no transcript could be detected was assigned a CT of 50 (the maximum cycle tested) if the housekeeping genes gave a positive result. This kept the graphs from being skewed due to missing data. Values were then plotted against minimum development percents. Binary expression plots were placed next to standard plots for a locus (no transcript results were removed). Left-right panel

comparisons demonstrated an increase in the standardized CT value in the right panels at points where gene expression was absent.

GAMs (Hastie and Tibshirani 1990; Wood 2006) were developed to assess the effects of gene expression (quantitative and binary data), body size, developmental stage, temperature, and strain on predicting minimum development percent. Each variable was assessed on its own, or together with only the non-linear data (the stage, temperature, and strain variables were only included if the variable was not a significant predictor of age on its own), to determine its usefulness in predicting blow fly age. The variables were used in larger models to determine the best GAMs for predicting blow fly age (Table 3). The models construct smoothing curves for non-linear data, which are cumulatively used to predict the age of a fly. However, the distribution of data around that smoothed curve must also be identified. The distribution is applied to the smoothed curve via a 'link function', thus each model was assigned a distribution and link function based on the criteria discussed in Generalized Additive Models. GAMs produce generalized cross validation (GCV) and percent deviance explained (PDE) statistics, which are explained in Generalized Additive Models. The significance of specific terms in the models was also estimated. Genes were considered candidate predictors of age if the estimated P-value associated with the individual gene by itself, or in combination with developmental stage and/or temperature, was below 0.025–0.008 (depending on the Bonferroni correction required). Once individual genes were determined to be likely predictors of development percent, they were used in combination with other such terms in larger GAMs. Ultimately, comparisons of the GCV and PDE scores from the resultant models helped to determine if and how gene expression was useful in predicting blow fly age. Diagnostic plots of error in the models were also constructed and are explained in Generalized Additive Models.

Results

Gene Sequences.

Fly strains were identified as *L. sericata* as noted in Generalized Additive Models. The sequenced genes underwent a BLAST search on the NCBI website (www.ncbi.nlm.nih.gov) (Table 2). All exhibited a close match to the same gene in another fly species. Two genes also demonstrated 99–100% matches to *L. sericata* sequences that were published after this project commenced.

Quantitative PCR and Statistical Modeling.

Of the 2559 flies detailed in Generalized Additive Models, 1025 were used to produce gene expression profiles. Once ~100 samples had been profiled, preliminary plots of the data indicated that *wg*, *scl*, and *rh3* were unlikely to provide useful information (Figures 1–3) and were dropped from further study. 958 of the samples yielded partial or full profiles of the remaining 9 genes. These were comprised of 48 first instar larvae, 79 second instar larvae, 135 third instar larvae, 334 postfeeding third instar larvae, and 362 pupae. There were 260 and 272 individuals profiled from the CA and MI strains (respectively) raised in the 20ºC treatments. Likewise 149, 121, and 156 individuals from the CA, MI, and WV strains (respectively) were profiled from the 33.5ºC treatments. Full gene expression profiles were obtained for 501 samples.

Plots were constructed to show the expression levels of each gene by developmental stage and by development percent. GAMs were used to assess the usefulness of individual gene expression profiles (quantitative and binary) for predicting blow fly age. Table 3 lists all GAMs investigated, with models 2–14 assessing the relative usefulness of individual genes. Table 4

lists the significance and degrees of freedom for terms in the models. The results of GAMs and plots for individual genes are listed below (Tables 3–4; Figures 1–39).

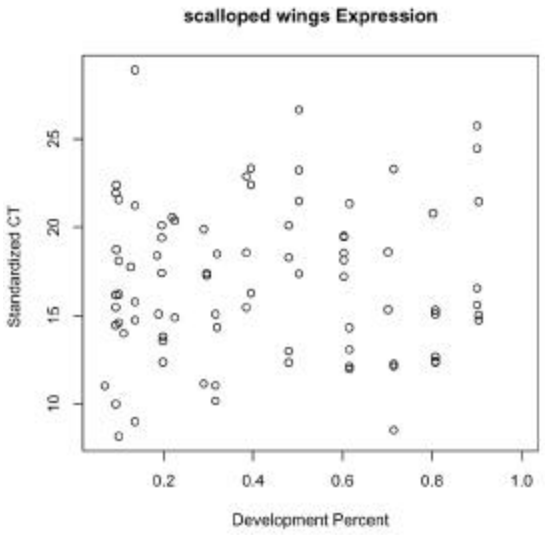

Figure 1. Standardized gene expression of *scalloped wings* throughout the immature development of *L. sericata.* No apparent pattern of gene expression was observed throughout development.

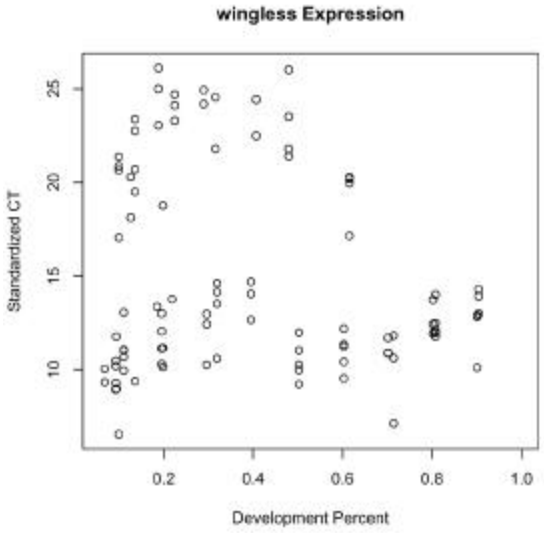

Figure 2. Standardized gene expression of *wingless* throughout the immature development of *L. sericata.* No informative pattern of gene expression was observed throughout development.

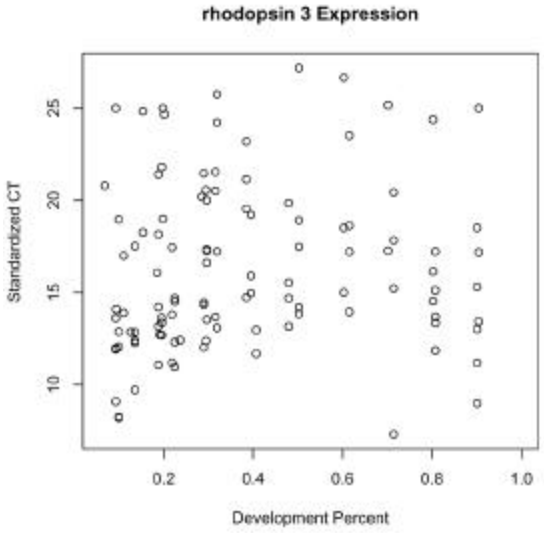

Figure 3. Standardized gene expression of *rhodopsin 3* throughout the immature development of *L. sericata.* No apparent pattern of gene expression was observed throughout development.

Gene Expression of Informative Genes

The most genes for forensic purposes are those that have stark changes in regulation during development, particularly the latter portion of development, where aging flies is most difficult. *cs*, *ecr*, *hsp60*, *hsp90*, *rop-1*, *w*, and *usp* were up- or down-regulated between feeding and postfeeding third instars. The most informative overall were *cs* and *ecr*. *cs* was strongly down-regulated during the postfeeding third instar, and it was often not expressed at all (or expressed at an undetectable level). Conversely, *ecr* was highly up-regulated during the postfeeding stage. The reliable expression differences of *cs*, *ecr*, and, to a lesser extent, the other genes noted above are useful indicators of the postfeeding condition and can be used as molecular markers for that stage. The overall behavior of each gene was as follows.

The first gene evaluated was *cs (*Figures 4–7; note throughout that higher CT scores indicate lower gene expression levels). Of the 958 individuals assessed, *cs* was not detected in 102. The gene was expressed at consistent levels, except during the postfeeding third instar, when it was expressed at lower levels (Figure 4) or not at all. *cs* tended to be expressed at higher levels at lower temperatures (Figure 5), with the MI strain expressing less *cs* at lower temperatures than the CA strain (Figure 6), while no expression level differences were apparent among the three strains at the high temperature treatment (Figure 7). The CA strain exhibited a stronger tendency to not express *cs* during the postfeeding third instar at 33.5ºC (Figure 7). GAMs assessing age with CT scores for this gene were only significant predictors of development percent when included with developmental stage (Table 4). The GAM that included the binary *cs* term (Model 2) exhibited a 0.3% higher PDE than the GAM that assessed age in terms of stage alone (Model 1), while the quantitative data (Model 6) exhibited an increase of 1.6% in PDE (Table 3).

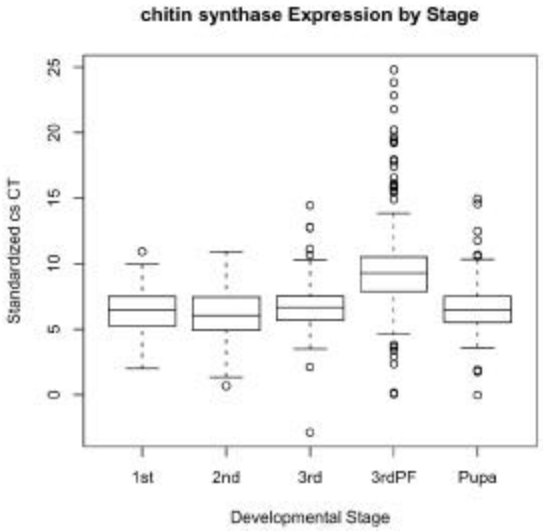

Figure 4. Standardized *chitin synthase* CT scores plotted by stage. The postfeeding third instar stands out as expressing significantly less *cs* than the other stages.

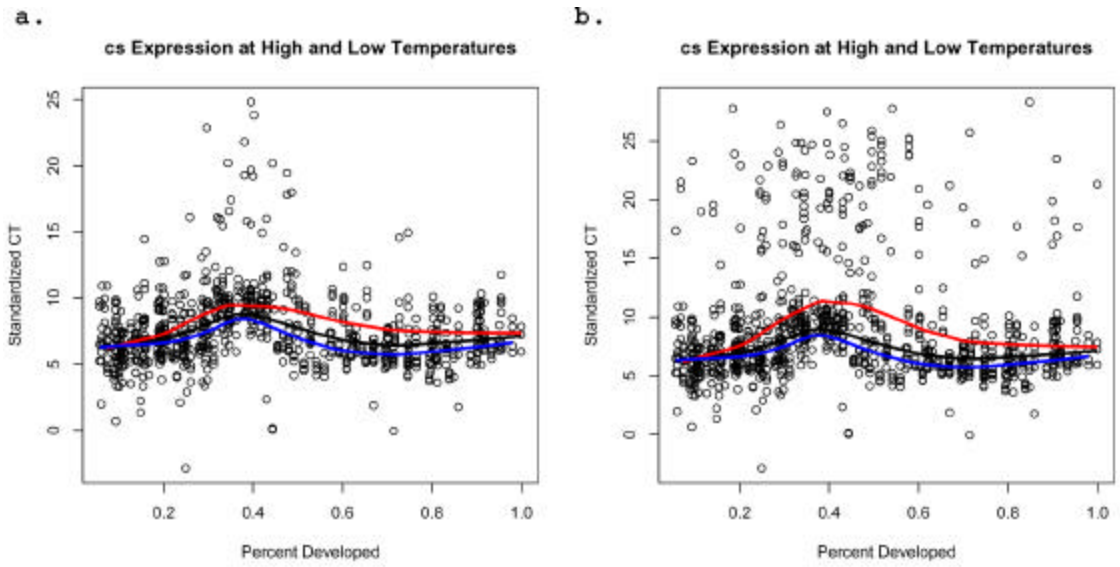

Figure 5. Standardized *chitin synthase* CT scores plotted by development percent for (a) all individuals that expressed the gene and (b) in all 958 individuals, with NA expression values assigned a CT of 50. The black line is the lowess curve for all individuals, the blue line is the curve for expression at 20ºC, and the red line is the curve for expression at 33.5ºC. The shift in the red line between (a) and (b) indicates a cluster of individuals that did not express the gene at the time point where gene expression is at its lowest levels for this locus.

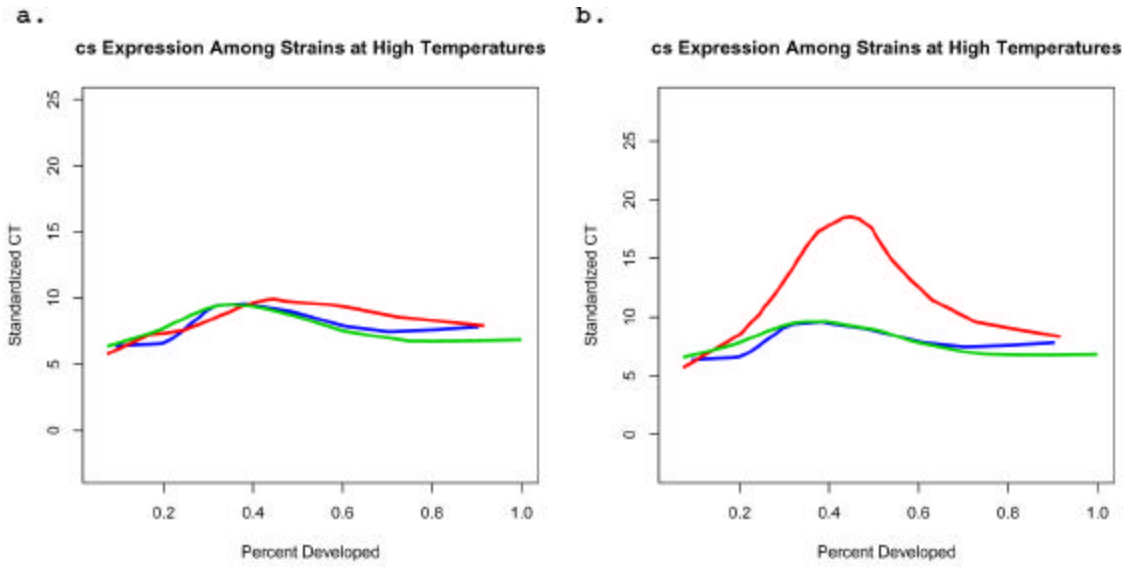

Figure 6. Standardized *chitin synthase* CT scores plotted by development percent at 33.5ºC for (a) all individuals that expressed the gene and (b) in all 958 individuals, with NA expression values assigned a CT of 50. The red line indicates gene expression in the CA strain. Blue indicates gene expression in the MI strain. The green line indicates gene expression in the WV strain. The shift in the red line between (a) and (b) indicates a cluster of individuals that did not express the gene at that time point.

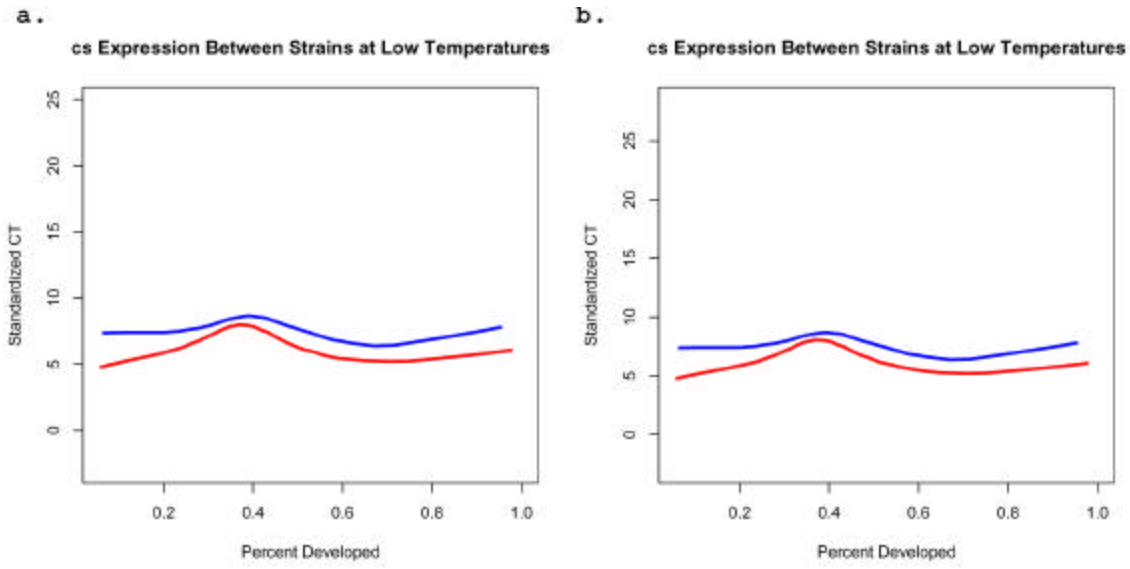

Figure 7. Standardized *chitin synthase* CT scores plotted by development percent at 20ºC for (a) all individuals that expressed the gene and (b) in all 958 individuals, with NA expression values assigned a CT of 50. The red line indicates gene expression in the CA strain. Blue indicates gene expression in the MI strain. Few individuals did not express the gene at this temperature. The CA strain expressed more of *cs* than the MI strain, though in the same pattern.

Plots of *hsp60* expression are in Figures 8–11. *hsp60* was not detected in 106 samples. When grouped by stage, *hsp60* was expressed at its highest levels in the first two instars, at intermediate levels during the feeding portion of the third instar, and at its lowest levels during the postfeeding third instar and pupal stages (Figure 8). When plotted against development percent, *hsp60* expression decreased from hatching until early pupation, and then increased in abundance until eclosion. Expression of *hsp60* was not affected by temperature (Figure 9). However, the CA strain expressed the gene at higher levels, though in the same pattern, than the MI strain at 20ºC (Figure 11). While the sample size was small, at 33.5ºC there was no obvious difference in expression among the strains, but the CA strain did express the gene at relatively high levels around 40 percent development (Figure 10). The gene was a significant predictor of development percent in the GAM assessing its utility (Table 4). The GAM that included the *hsp60* term (Model 7) exhibited a PDE of 14.7% (Table 3).

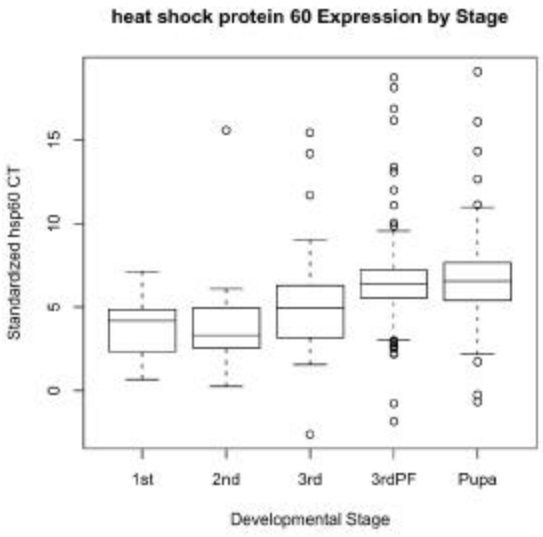

Figure 8. Standardized *heat shock protein 60* CT scores plotted by stage. Gene expression was highest during the first two stages and lowest during the last two stages.

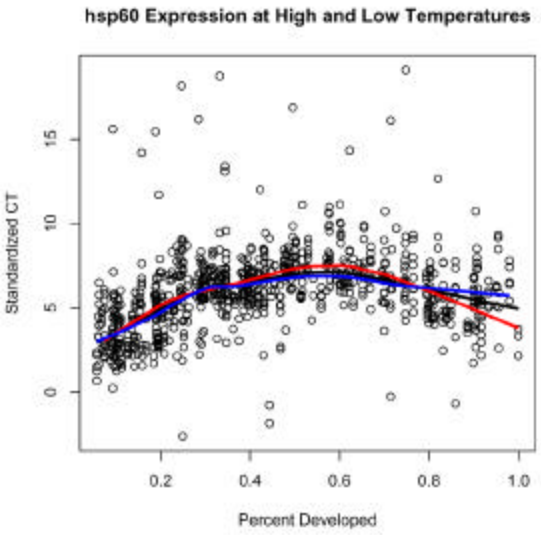

Figure 9. Standardized *heat shock protein 60* CT scores plotted by development percent for all individuals that expressed the gene. The black line is the lowess curve for all individuals, the blue line is the curve for expression at 20ºC, and the red line is the curve for expression at 33.5ºC. The expression levels of this gene decrease from hatching, until 60 percent development (early pupation), then increase until eclosion.

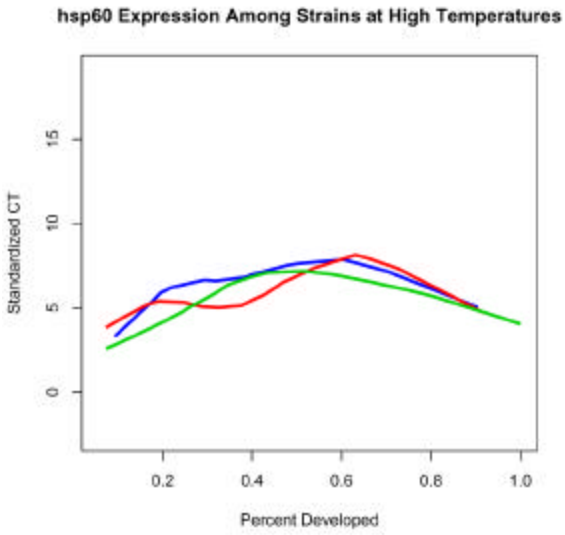

Figure 10. Standardized *heat shock protein 60* CT scores plotted by development percent at 33.5ºC for all individuals that expressed the gene. The red line indicates gene expression in the CA strain. Blue indicates gene expression in the MI strain. The green line indicates gene expression in the WV strain. The strains expressed the gene in the same general pattern, though the CA strain had higher expression around 40 percent development.

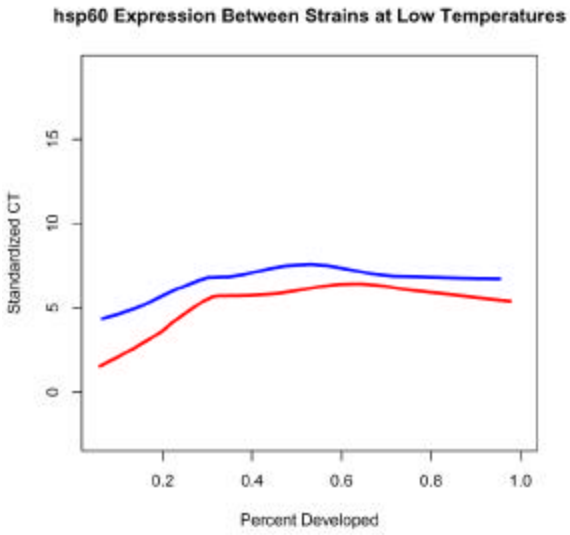

Figure 11. Standardized *heat shock protein 60* CT scores plotted by development percent at 20ºC for all individuals that expressed the gene. The red line indicates gene expression in the CA strain. Blue indicates gene expression in the MI strain. The CA strain expressed more of this gene than the MI strain, at this temperature. Few individuals did not express the gene at this temperature.

Plots of *hsp90* expression are in Figures 12–15. *hsp90* was not detected in 11 samples. When grouped by stage, *hsp90* was expressed at stable levels except during the feeding portion of the third instar, when it was expressed at lower levels than the other stages (Figure 12). When plotted against development percent, *hsp90* expression demonstrated different expression patterns depending on the temperature at which individuals were raised (Figure 13). During the first quarter of development (approximately) there was a decrease in transcript abundance, which was much more pronounced at 33.5ºC than at 20ºC. At the high temperature transcript abundance increases until eclosion, but at the lower temperature expression was maintained at a constant level. At 33.5ºC, there was no obvious difference in expression among strains, though the point of minimal *hsp90* expression occurred at a later time in the CA strain than it did in the other two (Figure 14). At 20ºC both strains demonstrated a similar pattern of *hsp90* expression, with the CA strain expressing the gene at elevated levels. The gene was a significant predictor of

development percent when included in a GAM assessing development percent with CT scores for *hsp90* along with temperature and developmental stage, which were also significant terms in that model (Table 4). The GAM (Model 8) exhibited a PDE increase of 0.6% compared to Model 1 (Table 3).

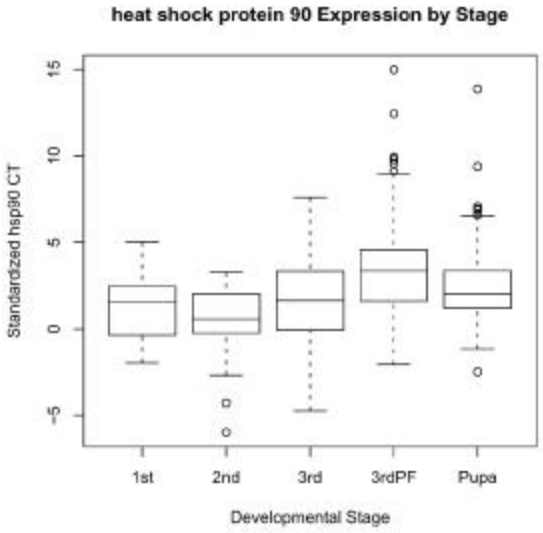

Figure 12. Standardized *heat shock protein 90* CT scores plotted by stage. Gene expression was lowest during the postfeeding third instar.

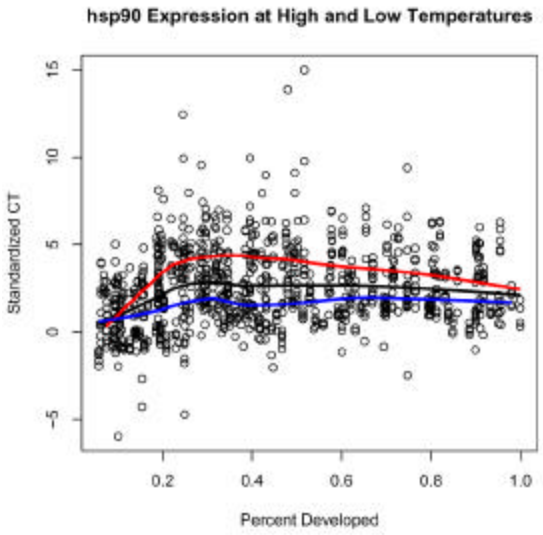

Figure 13. Standardized *heat shock protein 90* CT scores plotted by development percent for all individuals that expressed the gene. The black line is the lowess curve for all individuals, the blue line is the curve for expression at 20ºC, and the red line is the curve for expression at 33.5ºC. The expression levels of the gene decreased from hatching, until approximately 25 percent development (when postfeeding larval development is attained), then increased until eclosion when flies were raised at the high temperature. At the low temperature, expression was higher than at high temperatures, and expression was maintained at a steady level after 25 percent development.

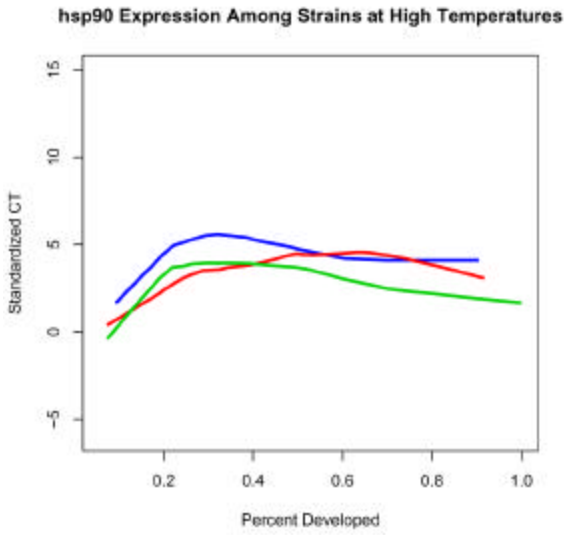

Figure 14. Standardized *heat shock protein 90* CT scores plotted by development percent at 33.5ºC for all individuals that expressed the gene. The red line indicates gene expression in the CA strain. Blue indicates gene expression in the MI strain. The green line indicates gene expression in the WV strain. Expression decreased until the postfeeding third instar (around 25 percent) then increased until eclosion. The strains expressed the gene in the same general pattern, though the time of minimum expression occurred later in the CA strain.

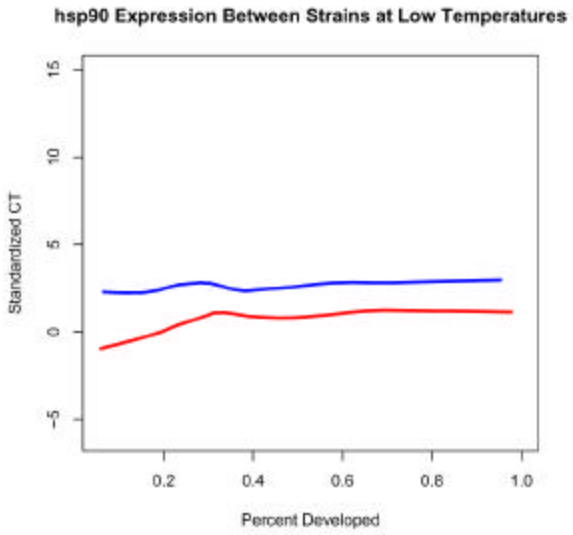

Figure 15. Standardized *heat shock protein 90* CT scores plotted by development percent at 20ºC for all individuals that expressed the gene. The red line indicates gene expression in the CA strain. Blue indicates gene expression in the MI strain. The CA strain expressed more of this gene than the MI strain, at this temperature.

Plots of *ace* expression are in Figures 16–19. *ace* was not detected in 322 samples. When grouped by stage, *ace* was expressed in decreasing levels through the third instar, and then it increased in expression until eclosion (Figure 16). When plotted against development percent, *ace* expression demonstrated two different expression patterns depending on the temperature at which individuals were raised (Figure 17). During the first quarter of development (approximately) there was a decrease in transcript abundance, which was much more pronounced at 33.5ºC than at 20ºC. At 33.5ºC transcript abundance increased until eclosion, but at the lower temperature expression was maintained at a constant level. At 33.5ºC there was no obvious difference in expression among the strains. Though sample sizes per strain were small, the point of minimal *ace* expression occurred at a later time in the CA strain than it did in the other two strains (Figure 14). In the 20ºC replicates, both strains demonstrated a similar pattern of expression, with the CA strain expressing the gene at elevated levels. In a GAM (Model 9), *ace*

was a significant predictor of development percent when temperature and developmental stage were included in the model. The binary term was significant by itself (Table 4). The non-parametric GAM (Model 9) exhibited a PDE increase of 1.7% compared to Model 1 (Table 3). The GAM that included binary *ace* expression (Model 3) demonstrated a PDE of 3.6% (Table 3).

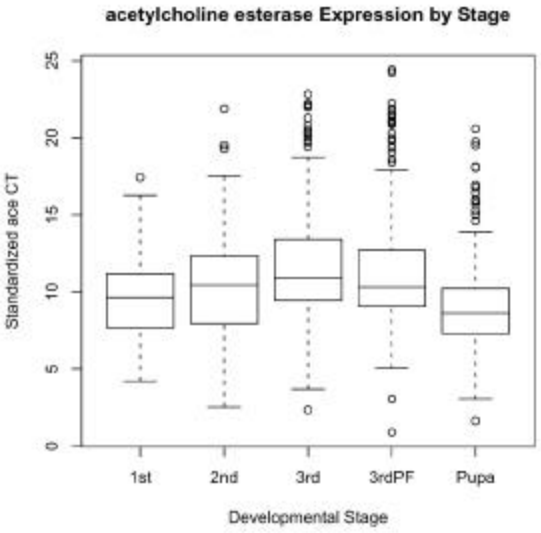

Figure 16. Standardized *acetylcholine esterase* CT scores plotted by stage. Gene expression decreased through the third instar, then increased until eclosion.

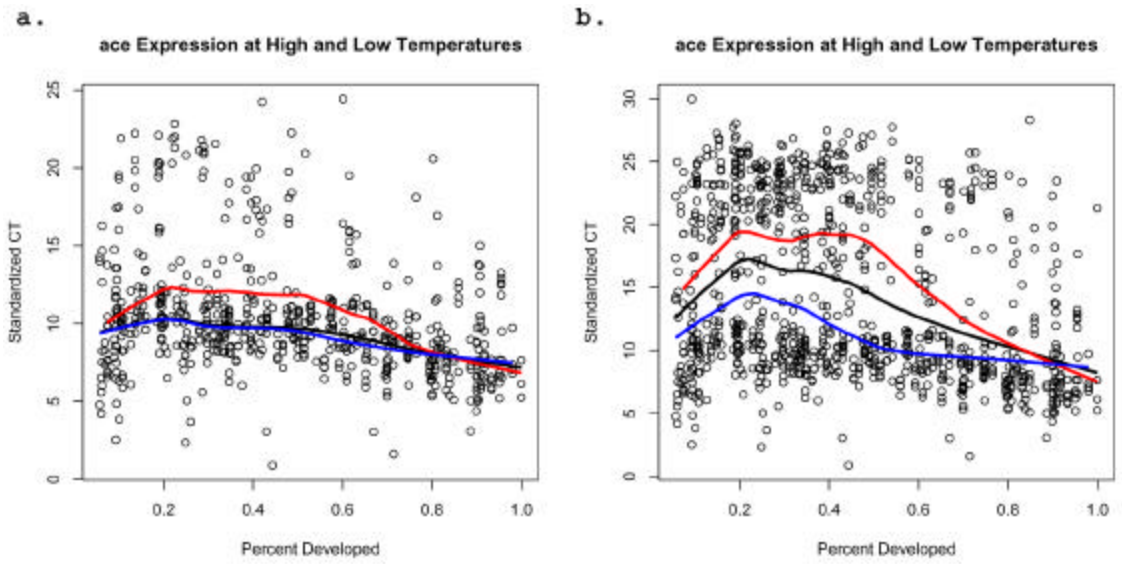

Figure 17. Standardized *acetylcholine esterase* CT scores plotted by development percent for (a) all individuals that expressed the gene and (b) in all 958 individuals, with NA expression values assigned a CT of 50. The black line is the lowess curve for all individuals, the blue line is the curve for expression at 20ºC, and the red line is the curve for expression at 33.5ºC. The shift in the lines between (a) and (b) indicates a cluster of individuals that do not express the gene at the time point where gene expression is at its lowest levels for this locus. Many individuals did not express *ace* between approximately 20 and 50 percent development (late third instar through early pupation). This time period mirrors the period of least *ace* expression at the high temperature treatment. Expression increased through development in the low temperature treatments.

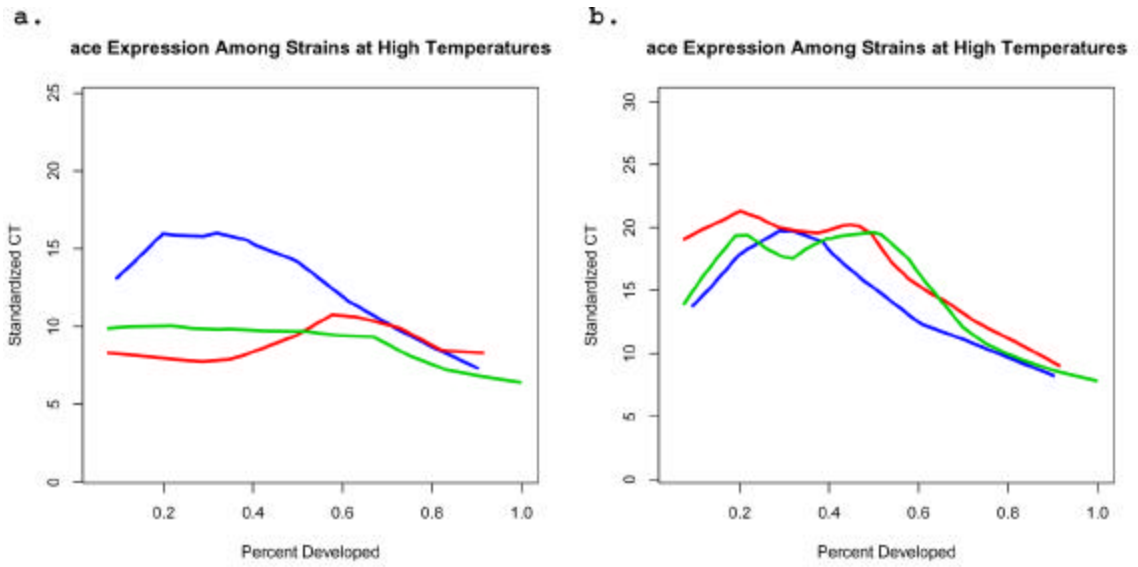

Figure 18. Standardized *acetylcholine esterase* CT scores plotted by development percent at 33.5ºC for (a) all individuals that expressed the gene and (b) in all 958 individuals, with NA expression values assigned a CT of 50. The red line indicates gene expression in the CA strain. Blue indicates gene expression in the MI strain. The green line indicates gene expression in the WV strain. The shift in these lines between (a) and (b) indicates a cluster of individuals that do not express the gene at that time point.

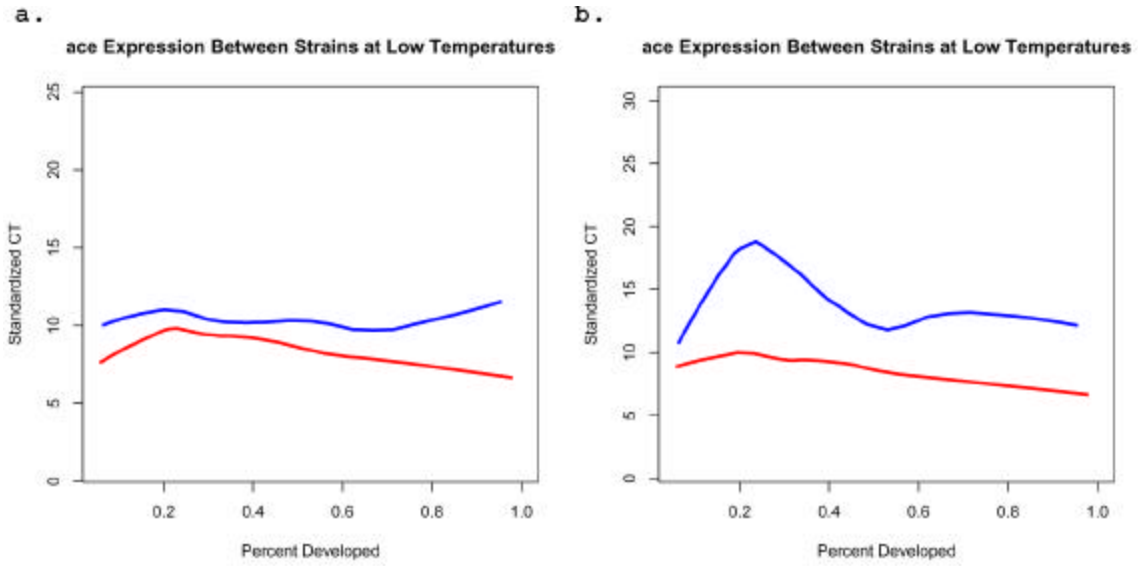

Figure 19. Standardized *acetylcholine esterase* CT scores plotted by development percent at 20ºC for (a) all individuals that expressed the gene and (b) in all 958 individuals, with NA expression values assigned a CT of 50. The red line indicates gene expression in the CA strain. Blue indicates gene expression in the MI strain. The CA strain expressed more *ace* than the MI strain (a). MI was also more likely to not express *ace*.

Plots of *ecr* expression are in Figures 20–23. *ecr* was not detected in 17 samples. When grouped by stage, *ecr* was expressed in slightly increasing levels until the postfeeding third instar, when expression was significantly greater than other stages, then expression decreased through pupation until eclosion (Figure 20). When plotted against development percent, *ecr* expression increased until achieving maximum expression at approximately 35 percent, then expression decreased. This pattern was the same for cohorts raised at both temperatures (Figure 21). However, at 33.5ºC, the MI strain expressed less *ecr* than the other strains (Figure 22) and at 20ºC the MI strain expressed less *ecr* than the CA strain, except at the point of maximum expression, when both strains converged to the same expression level for this gene (Figure 23). A GAM assessing age with *ecr* CT scores was a significant predictor of development percent (Table 4). This GAM (Model 10) exhibited a PDE of 6.6% (Table 3).

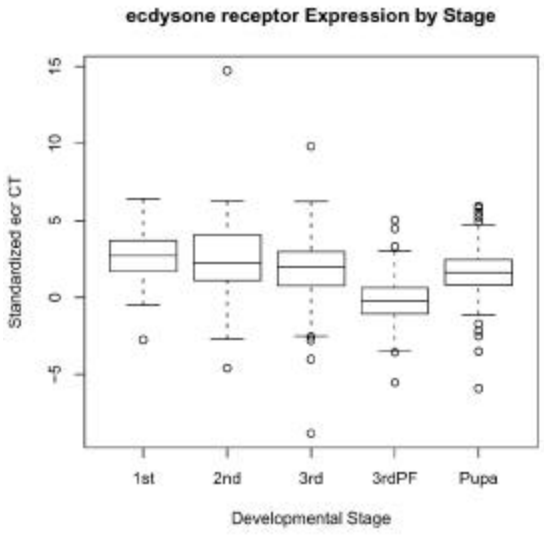

Figure 20. Standardized *ecdysone receptor* CT scores plotted by stage. Gene expression increased through the postfeeding third instar, then decreased until eclosion.

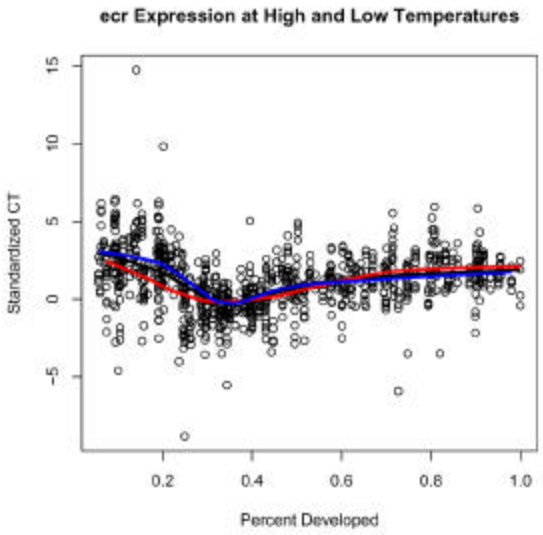

Figure 21. Standardized *ecdysone receptor* CT scores plotted by development percent for all individuals that expressed the gene. The black line is the lowess curve for all individuals, the blue line is the curve for expression at 20ºC, and the red line is the curve for expression at 33.5ºC. The expression levels of this gene increase from hatching, until approximately 35 percent development (during the postfeeding third instar), then decrease until eclosion.

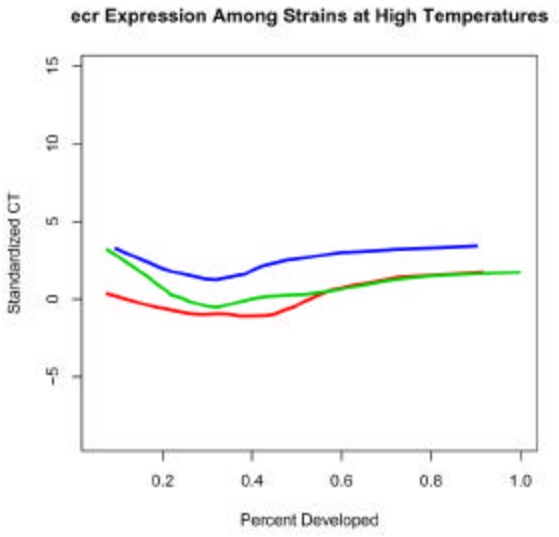

Figure 22. Standardized *ecdysone receptor* CT scores plotted by development percent at 33.5ºC for all individuals that expressed the gene. The red line indicates gene expression in the CA strain. Blue indicates gene expression in the MI strain. The green line indicates gene expression in the WV strain. The strains expressed the gene in the same general pattern, though the MI strain expressed less *ecr* than the other strains.

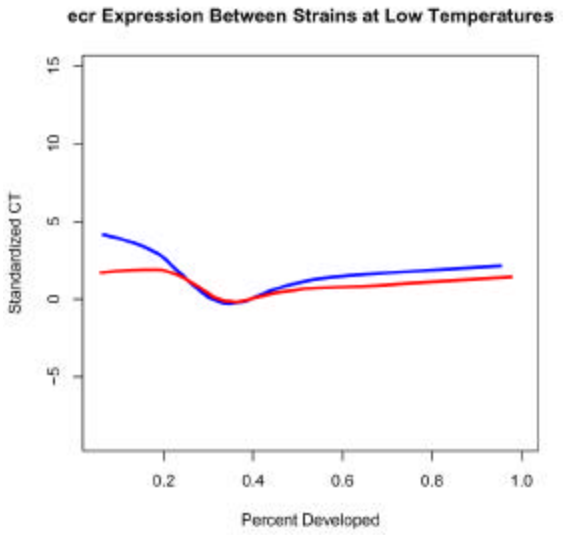

Figure 23. Standardized *ecdysone receptor* CT scores plotted by development percent at 20ºC for all individuals that expressed the gene. The red line indicates gene expression in the CA strain. Blue indicates gene expression in the MI strain. The CA strain expressed more of this gene than the MI strain, at this temperature, though they converge to the same maximum expression level during the postfeeding third instar.

Plots of *rop–1* expression are in Figures 24–27. Of the 958 individuals assessed, *rop–1* was not detected in 99 samples. When grouped by stage, *rop–1* was expressed in increasing levels through the postfeeding third instar, then expression decreased through pupation until eclosion (Figure 24). When plotted by development percent, *rop–1* increased in abundance until approximately 25 percent development (when raised at 33.5ºC), or until 35 percent development (when raised at 20ºC) (Figure 25). The shift in these curves closely mirrored the shift in body size (seen in Generalized Additive Models), which did not occur with all genes. After achieving maximum expression, the abundance of message decreased until eclosion. At high temperatures, there was no obvious difference in expression of *rop–1* among the strains (Figure 26). However, at the low temperature treatment, expression was consistently higher in the CA strain compared to the MI strain (Figure 27). In the GAM assessing age with *rop–1* CT scores, the gene was a significant predictor of development percent when included with stage (Table 4). Though both

terms were significant predictors of development percent, the GAM (Model 11) exhibited no PDE increase when compared to Model 1. However, the GCV score was lower for Model 11 when compared to Model 1 (Table 3), indicating that the inclusion of *rop–1* will help make better estimates of development percent.

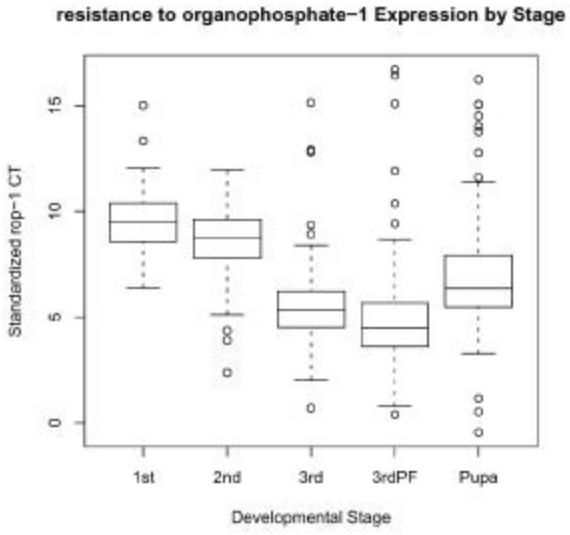

Figure 24. Standardized *resistance to organophosphate 1* CT scores plotted by stage. Gene expression increased through the postfeeding third instar, then decreased until eclosion.

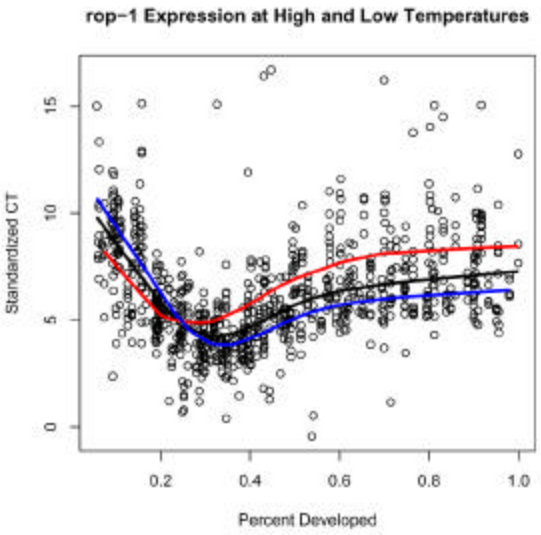

Figure 25. Standardized *resistance to organophosphate 1* CT scores plotted by development percent for all individuals that expressed the gene. The black line is the lowess curve for all individuals, the blue line is the curve for expression at 20ºC, and the red line is the curve for expression at 33.5ºC. The expression levels of this gene increase from hatching until approximately 25 percent development at high temperatures and until approximately 35 percent development at low temperatures, then gene transcript abundance decrease until eclosion.

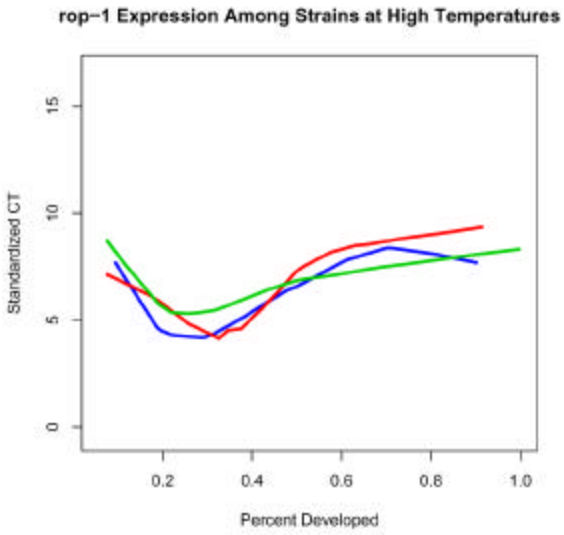

Figure 26. Standardized *resistance to organophosphate 1* CT scores plotted by development percent at 33.5ºC for all individuals that expressed the gene. The red line indicates gene expression in the CA strain. Blue indicates gene expression in the MI strain. The green line indicates gene expression in the WV strain. The strains expressed the gene in the same general pattern.

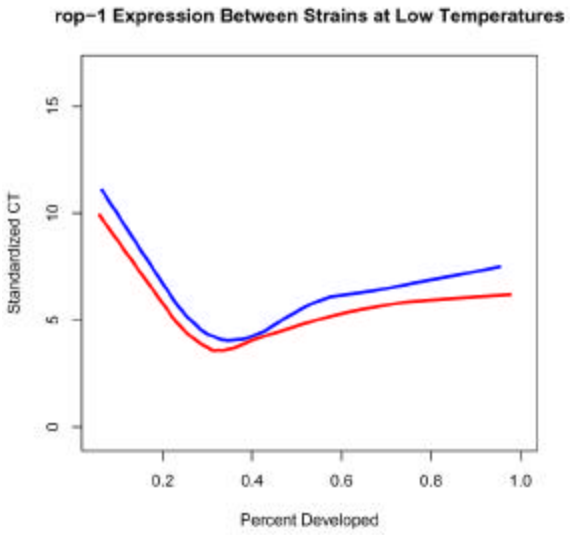

Figure 27. Standardized *resistance to organophosphate 1* CT scores plotted by development percent at 20ºC for all individuals that expressed the gene. The red line indicates gene expression in the CA strain. Blue indicates gene expression in the MI strain. The CA strain expressed more of the gene than the MI strain, at this temperature, though both followed the same pattern.

Plots of *w* expression are in Figures 28–31. *w* was not detected in 233 samples. When grouped by stage, *w* was expressed in increasing amounts from the first instar through the postfeeding third instar, then expression decreased in pupae (Figure 28). When plotted by development percent, *w* increased in abundance until approximately 25 percent development (when raised at 33.5ºC), or until 35 percent development (when raised at 20ºC) (Figure 29), though this pattern was subtler than in the *rop–1* plots. Individuals from the low temperature treatments were also more likely to express *w* than individuals from high temperature treatments (Figure 29). After achieving maximum expression, the abundance of the transcript decreased until eclosion. At high temperatures, there was no obvious difference in expression of *w* among the strains (Figure 30), but the CA strain was the least likely to express the gene at that temperature and the MI strain was the most likely to express it. However, at the low temperature

treatment, expression was consistently higher in the CA strain compared to the MI strain, with the MI strain likely to not express the gene during pupation (Figure 31). In GAMs assessing both the binary and non-parametric expression of *w*, the gene was a significant predictor of development percent (Table 4). Binary expression (Model 4) explained 0.43% of the deviance in the data. When *w* CT scores were used to predict development percent (Model 12), a PDE of 3.55% was attained (Table 3).

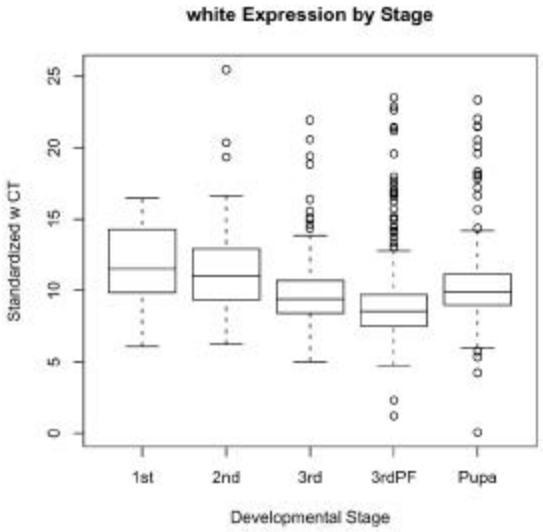

Figure 28. Standardized *white* CT scores plotted by stage. Gene expression increased through the postfeeding third instar, then decreased until eclosion.

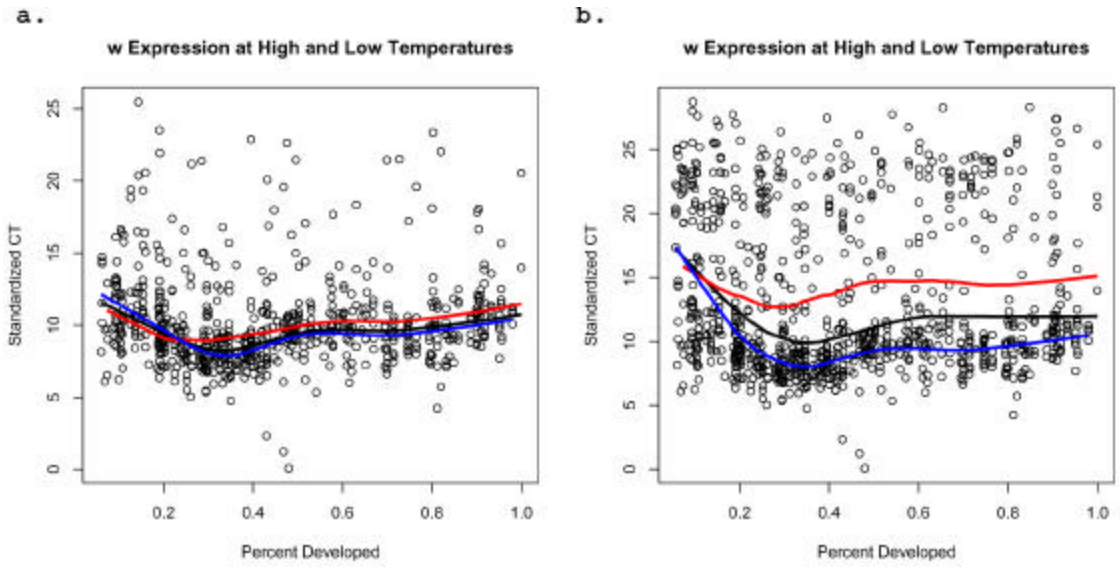

Figure 29. Standardized *white* CT scores plotted by development percent for (a) all individuals that expressed the gene and (b) in all 958 individuals, with NA expression values assigned a CT of 50. The black line is the lowess curve for all individuals, the blue line is the curve for expression at 20ºC, and the red line is the curve for expression at 33.5ºC. The shift in the lines between (a) and (b) indicates a cluster of individuals that did not express the gene at the time point where gene expression is at its lowest levels for this locus. The expression levels of the gene increased from hatching until approximately 25 percent development at high temperatures and until approximately 35 percent development at low temperatures, then gene transcript abundance decrease until eclosion. High temperature treatments were less likely to express the gene.

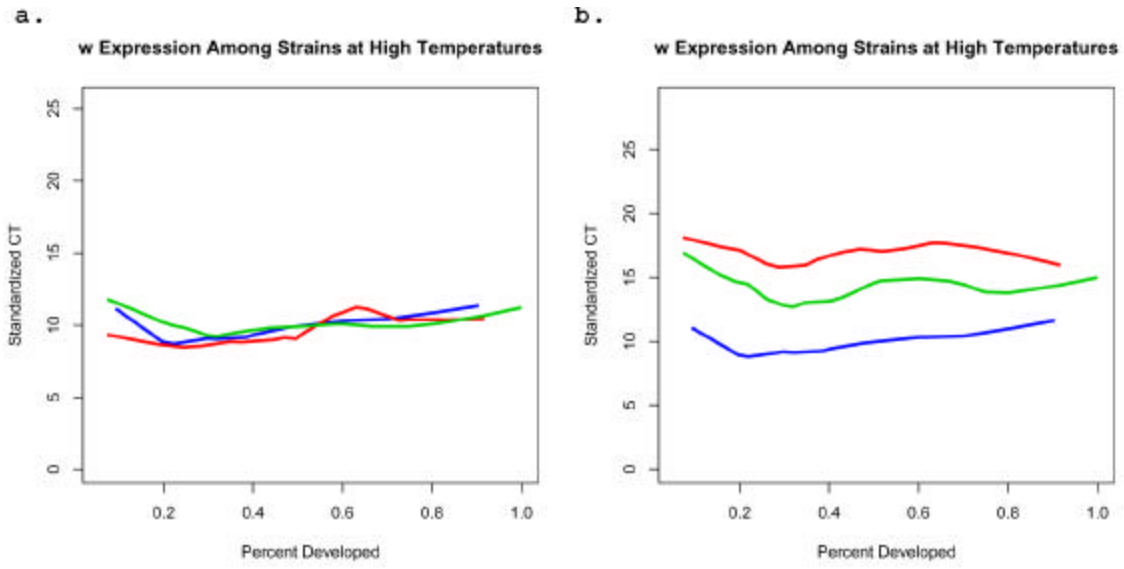

Figure 30. Standardized *white* CT scores plotted by development percent at 33.5ºC for (a) all individuals that expressed the gene and (b) in all 958 individuals, with NA expression values assigned a CT of 50. The red line indicates gene expression in the CA strain. Blue indicates gene expression in the MI strain. The green line indicates gene expression in the WV strain. The shift in the lines between (a) and (b) indicates that CA was most likely to not express *w* and MI was most likely to express the gene.

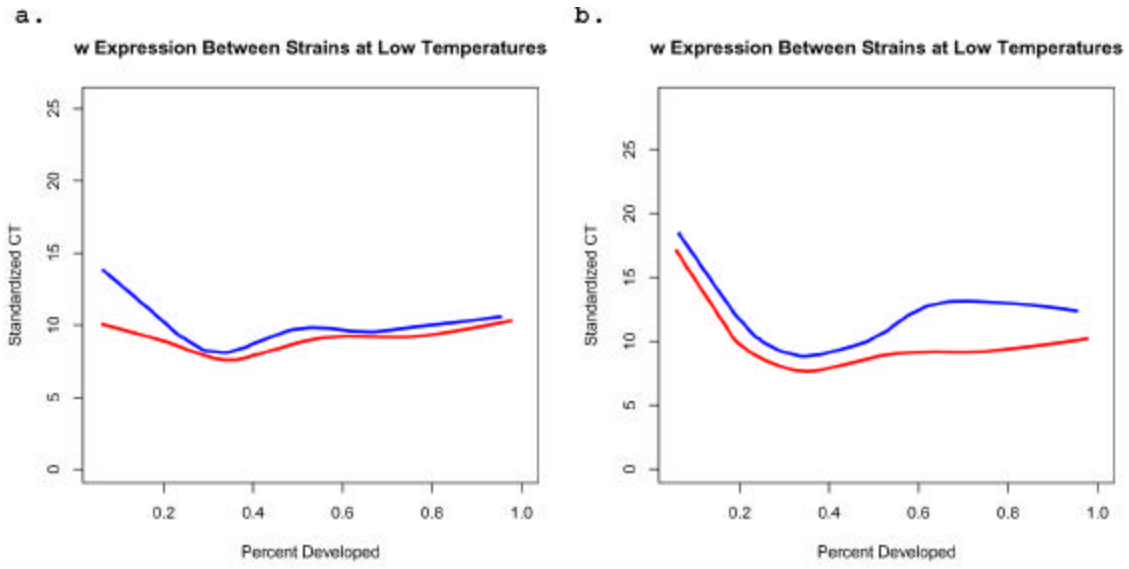

Figure 31. Standardized *white* CT scores plotted by development percent at 20ºC for (a) all individuals that expressed the gene and (b) in all 958 individuals, with NA expression values assigned a CT of 50. The red line indicates gene expression in the CA strain. Blue indicates gene expression in the MI strain. The CA strain expressed more *w* than the MI strain (a). MI was also more likely to not express *w* during pupation (the last half of development).

Plots of *usp* expression are in Figures 32–35. *usp* was not detected in 109 samples. When grouped by stage, *usp* was expressed in increasing amounts from the first instar through the postfeeding third instar, and then expression decreased in pupae (Figure 32). When plotted by development percent, *usp* increased in concentration from hatching until the postfeeding third instar, then expression decreased through pupation (Figure 33), with no obvious difference in expression among temperature treatments. The three strains exhibited little difference in expression at 33.5ºC (Figure 34). However, at 20ºC MI and CA expressed *usp* in a similar pattern, with CA consistently expressing more of the gene than the MI strain. A GAM assessing expression of *usp* was a significant predictor of development percent when stage was included in the model (Table 4). When *usp* CT scores were used to predict development percent (Model 13), a PDE increase of 0.6% was attained (Table 3).

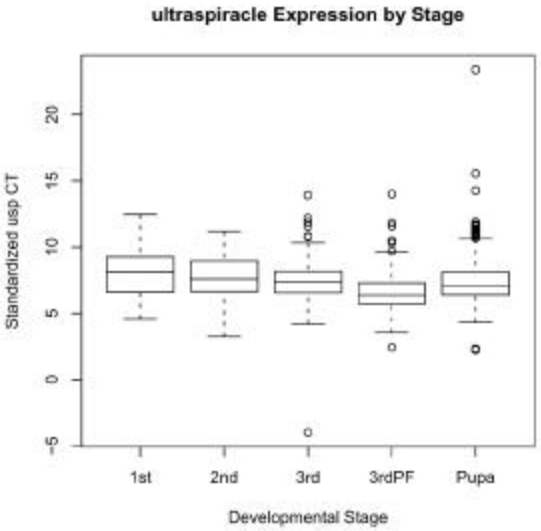

Figure 32. Standardized *ultraspiracle* CT scores plotted by stage. Gene expression increased through the postfeeding third instar, then decreased until eclosion.

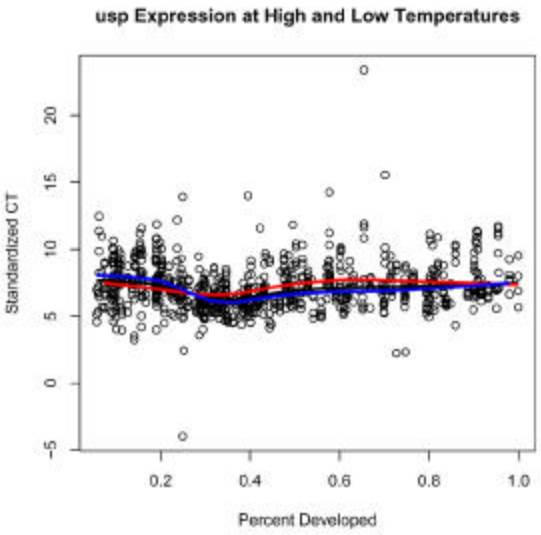

Figure 33. Standardized *ultraspiracle* CT scores plotted by development percent for all individuals that expressed the gene. The black line is the lowess curve for all individuals, the blue line is the curve for expression at 20ºC, and the red line is the curve for expression at 33.5ºC. The expression levels of the gene increased from hatching until approximately 35 percent development, then gene transcript abundance decreased until eclosion.

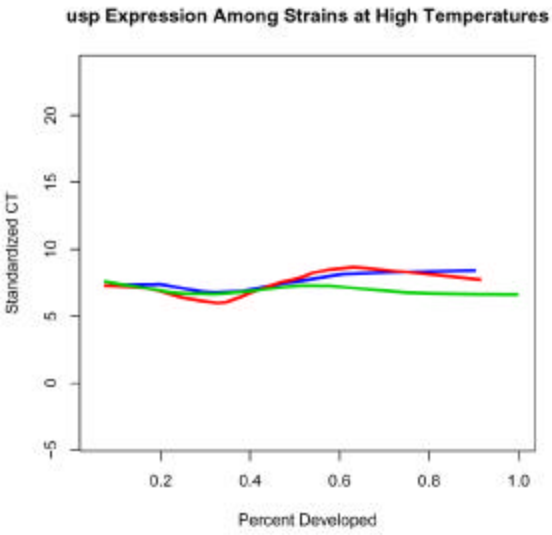

Figure 34. Standardized *ultraspiracle* CT scores plotted by development percent at 33.5ºC for all individuals that expressed the gene. The red line indicates gene expression in the CA strain. Blue indicates gene expression in the MI strain. The green line indicates gene expression in the WV strain. The strains expressed the gene in the same general pattern.

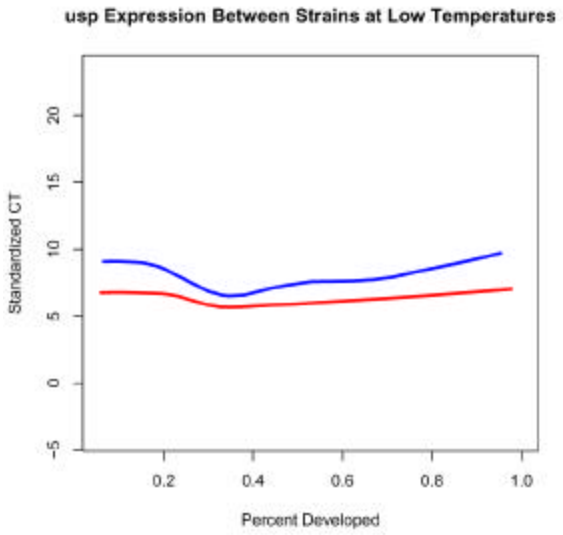

Figure 35. Standardized *ultraspiracle* CT scores plotted by development percent at 20ºC for all individuals that expressed the gene. The red line indicates gene expression in the CA strain. Blue indicates gene expression in the MI strain. The CA strain expressed more of the gene than the MI strain, though both followed a similar pattern.

Plots of *sll* expression are in Figures 36–39. Of the 958 individuals sampled, 281 did not express the gene. When plotted by stage, expression increased through the feeding portion of development, then decreased until eclosion (Figure 36). When plotted by development percent, expression increased until approximately 25 percent development, and then decreased until approximately 50 percent development, when expression levels increased again until eclosion (Figure 37). There was little difference in expression patterns between temperatures, but individuals from high temperature treatments were much less likely to express the gene (Figure 37). When expression at the high temperature was assessed, it was clear that the lack of expression occurred in both the CA and WV strains (Figure 38). The expression of *sll* at low temperatures was similar among the CA and MI strains, but MI was much less likely to express the gene (Figure 39). When GAMs utilized *sll* expression to predict development percent, both the binary and quantitative expression levels of the gene were significant predictors of age (Table

4). The GAM that utilized standardized *sll* CT scores (Model 14) exhibited a PDE of 9.17%. The GAM using the binary expression data (Model 5) required the assessment of stage, strain, and temperature with *sll* to achieve an increase of 0.3% in PDE compared to Model 1 (Table 3).

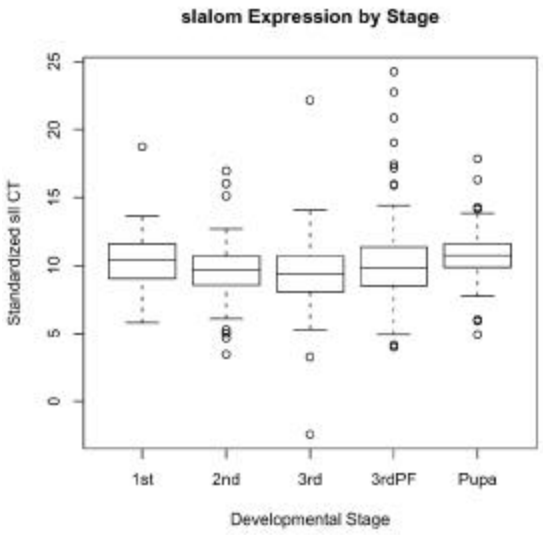

Figure 36. Standardized *slalom* CT scores plotted by stage. Gene expression increased through the third instar, then decreased until eclosion.

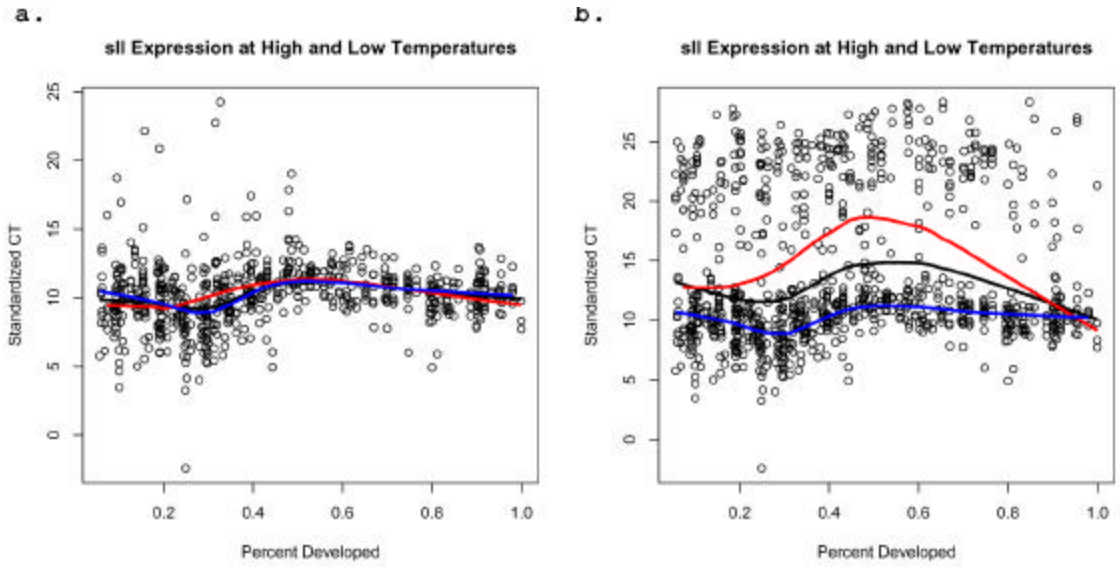

Figure 37. Standardized *slalom* CT scores plotted by development percent for (a) all individuals that expressed the gene and (b) in all 958 individuals, with NA expression values assigned a CT of 50. The black line is the lowess curve for all individuals, the blue line is the curve for expression at 20ºC, and the red line is the curve for expression at 33.5ºC. The shift in the lines between (a) and (b) indicates a cluster of individuals that did not express the gene at the time point where gene expression was at its lowest levels. The expression levels of the gene increased from hatching until approximately 25 percent development, then gene transcript abundance decreased until approximately 50 percent, and then increase again until eclosion. High temperature treatments were less likely to express the gene.

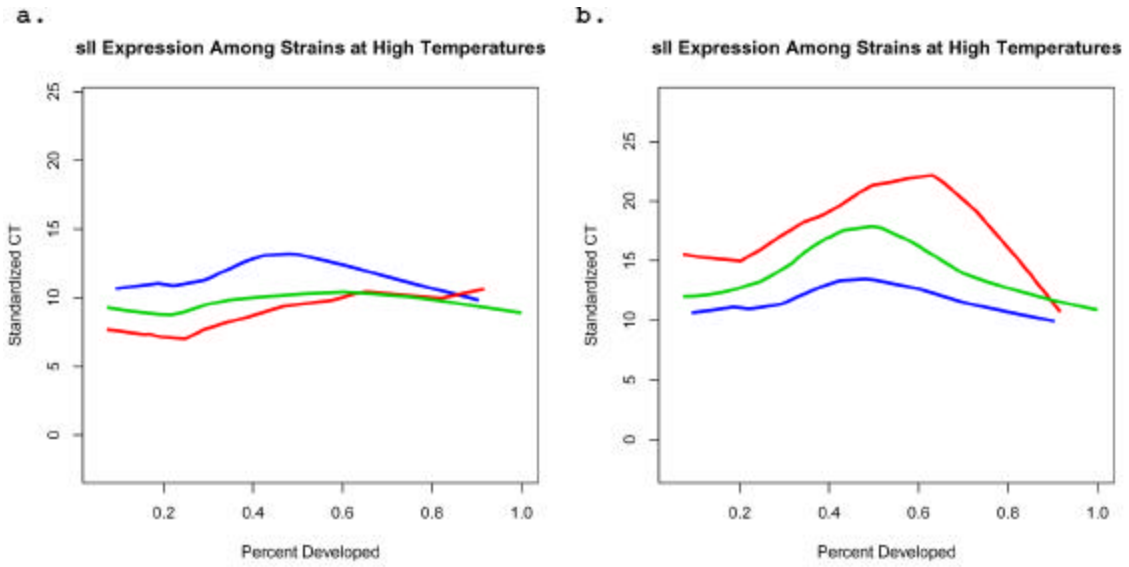

Figure 38. Standardized *slalom* CT scores plotted by development percent at 33.5ºC for (a) all individuals that expressed the gene and (b) in all 958 individuals, with NA expression values assigned a CT of 50. The red line indicates gene expression in the CA strain. Blue indicates gene expression in the MI strain. The green line indicates gene expression in the WV strain. The shift in the lines between (a) and (b) indicates that CA was most likely to not express *sll* and MI was most likely to express the gene.

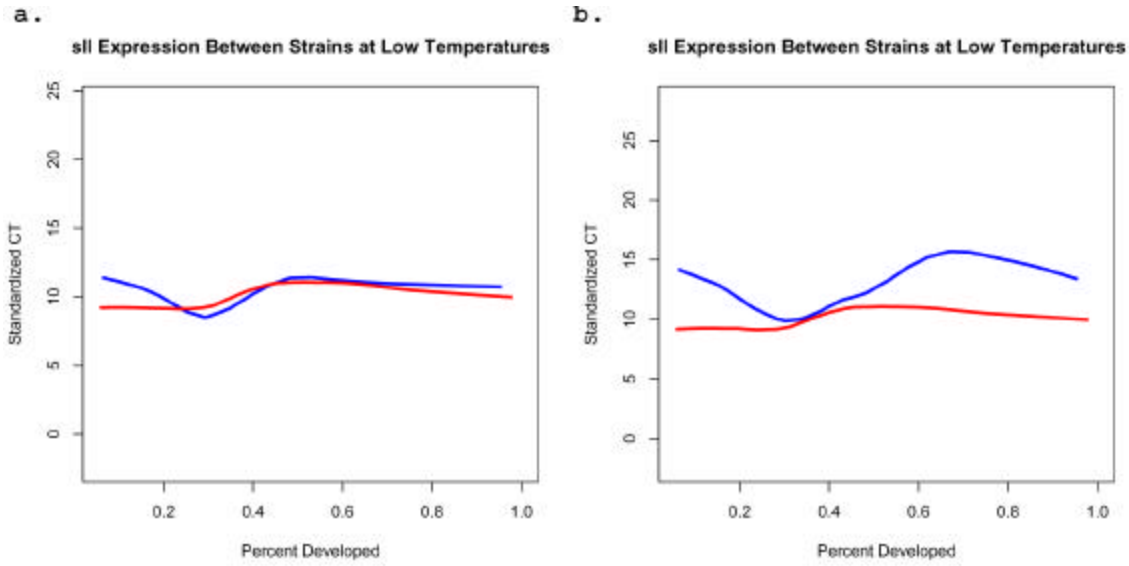

Figure 39. Standardized *slalom* CT scores plotted by development percent at 20ºC for (a) all individuals that expressed the gene and (b) in all 958 individuals, with NA expression values assigned a CT of 50. The red line indicates gene expression in the CA strain. Blue indicates gene expression in the MI strain. The strains expressed the gene in a similar pattern (a). MI was more likely to not express *sll*.

The expression profiles of all developmentally regulated genes are shown in Figure 40. All GAMs that included expression data from multiple genes (Models 16–23) were found to have lower GCV scores than the control models. Likewise, all but one model (Model 19 which used a gaussian distribution with gene expression data) exhibited an increase in PDE compared to the control models (92.1%– 95.7% PDE compared to 88.2%– 91.8%; Table 3). Model 19 had a PDE of 91.3%, which was 0.5% lower than Model 15 and 3.1% better than the PDE for Model 1. (See information on specific stages below.)

When diagnostic plots of the models were compared the improvement generated by incorporating gene expression was substantial. Figures 41–48 depict diagnostic plots, which assess error for specific models tested in this experiment. The control models (Figures 41 and 42) show non-gene expression data only, while the rest display control data and some form of gene expression data used to predict development percent. The lower right quadrant of the plots depicts the predicted (Fitted) versus true (Response) development percents of all individuals used to make the model. In model 15 (which used non-gene data, Figure 42), error increased throughout development and a gap existed between larval and pupal predictions. All gene expression models decreased error and closed the gap in predictions that were present in Figures 41 and 42.

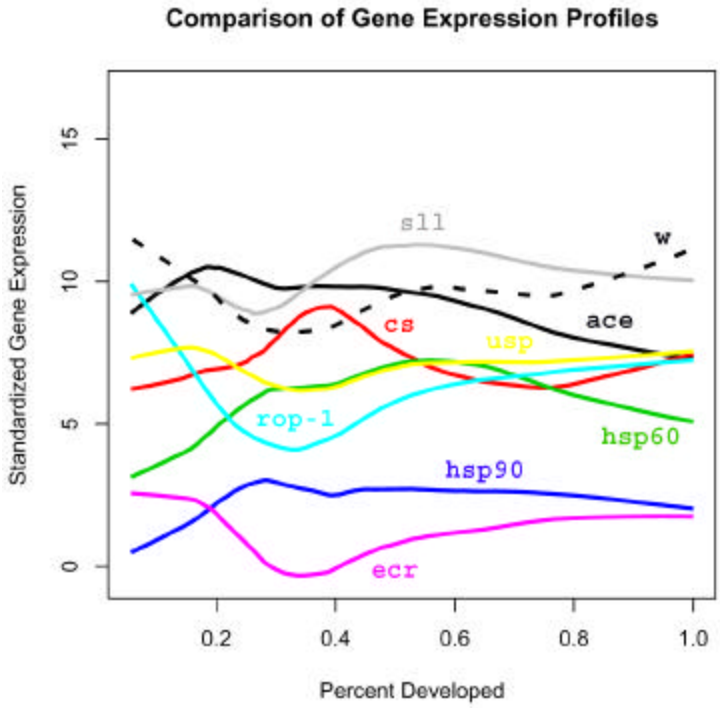

Figure 40. The combined standardized expression (CT score) patterns for all nine genes throughout *L. sericata* immature development. The lowess curves for each gene represent expression for all individuals that expressed the gene. The combined expression patterns of the genes were used to predict blow fly age with GAMs.

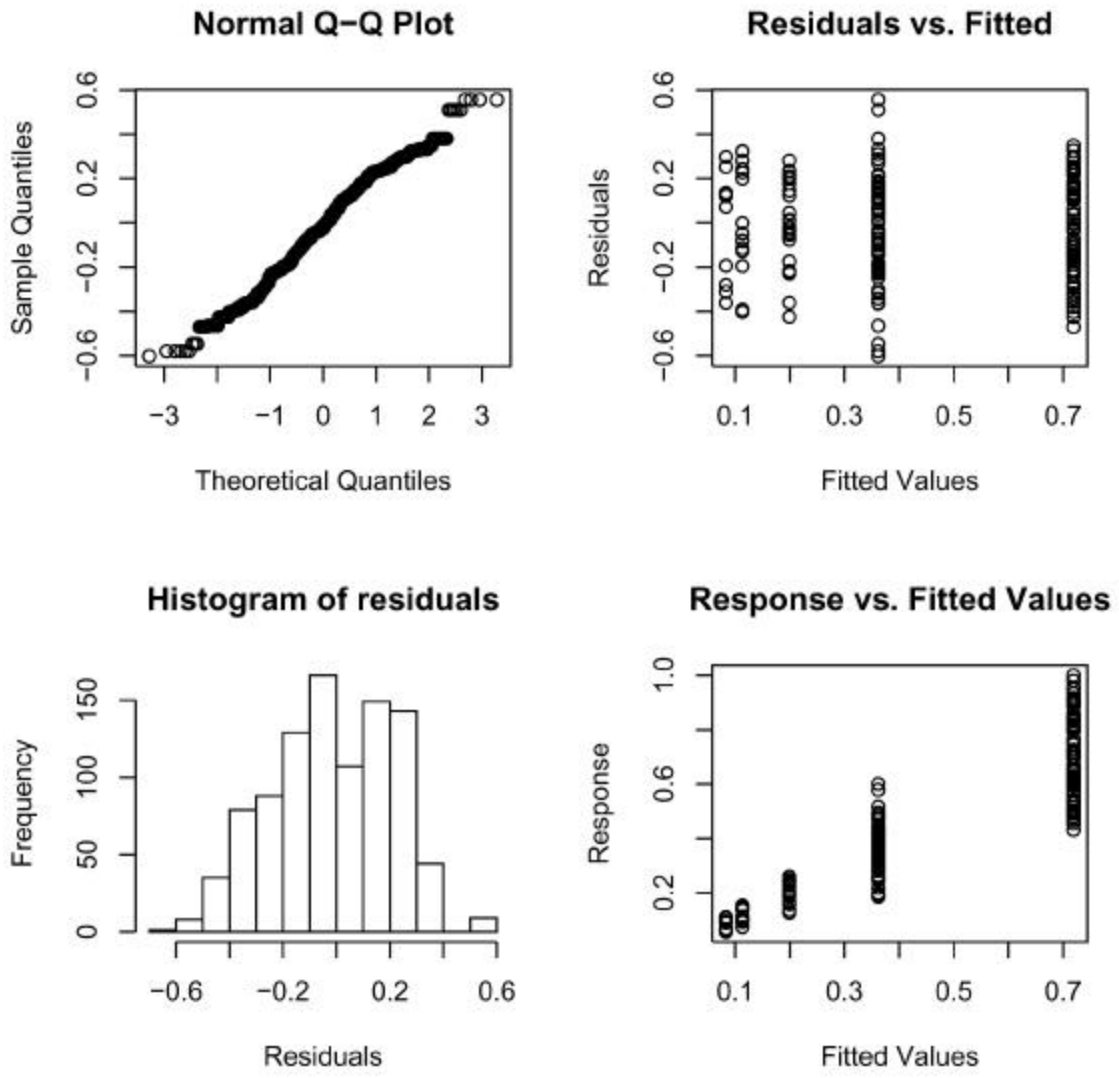

Figure 41. Diagnostic plot for Model 1, which used developmental stage to predict *L. sericata* development percent (on a scale of 0–1). A gamma distribution was used with this model. Descriptions for each panel type can be found in Figure 4 of Generalized Additive Models. Predicted (Fitted) values represent a range of true (Response) values. Error increased with age, and gaps exist between predictions for each developmental stage.

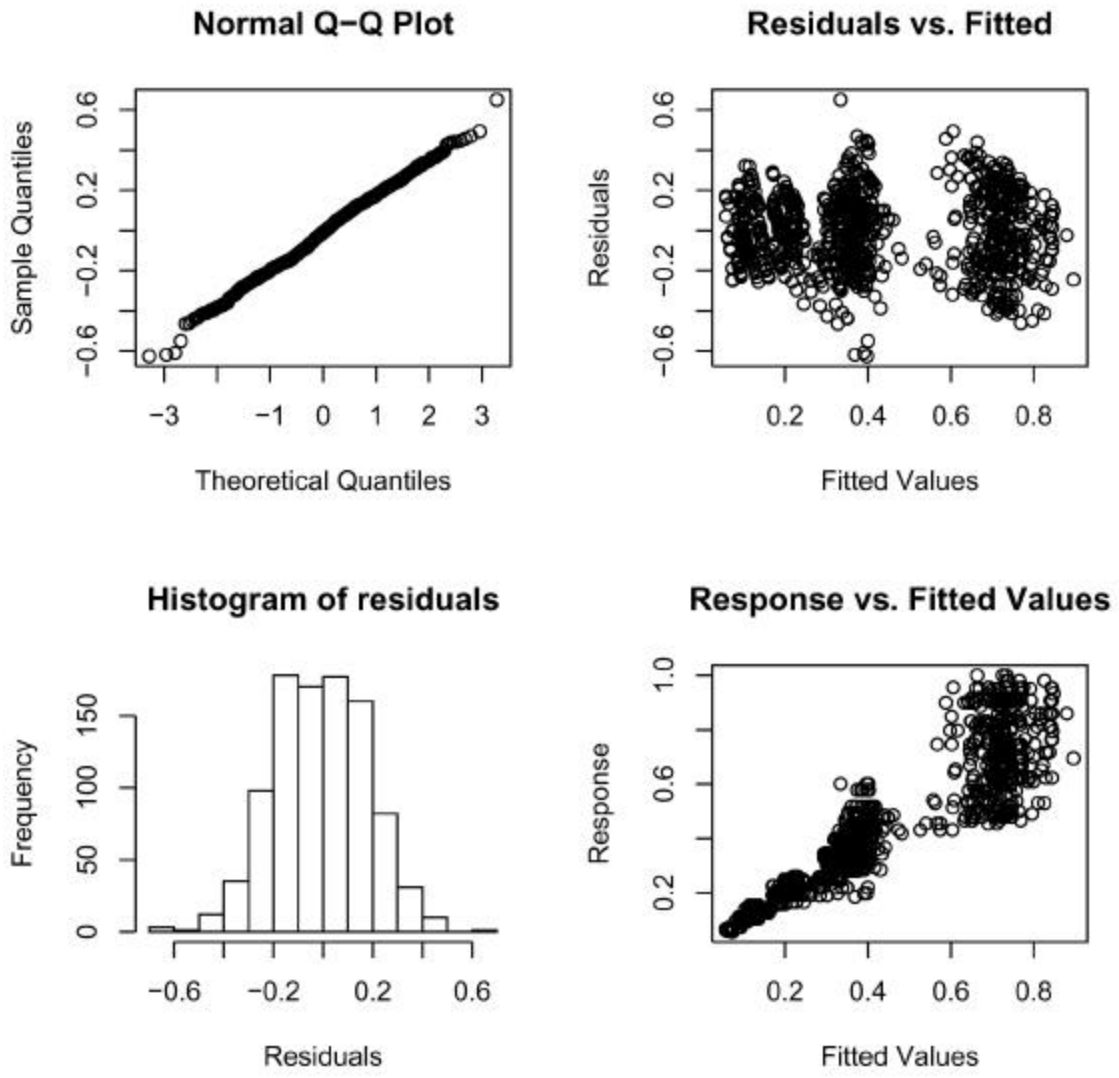

Figure 42. Diagnostic plot for Model 15, which used developmental stage, strain, temperature, length, and weight to predict *L. sericata* development percent (on a scale of 0–1). A gamma distribution was used with this model. Descriptions for each panel type can be found in Figure 4 of Generalized Additive Models. Predicted (Fitted) values represent a range of true (Response) values. Error increased with age, and a gap exists between predictions for postfeeding third instar and pupal ages.

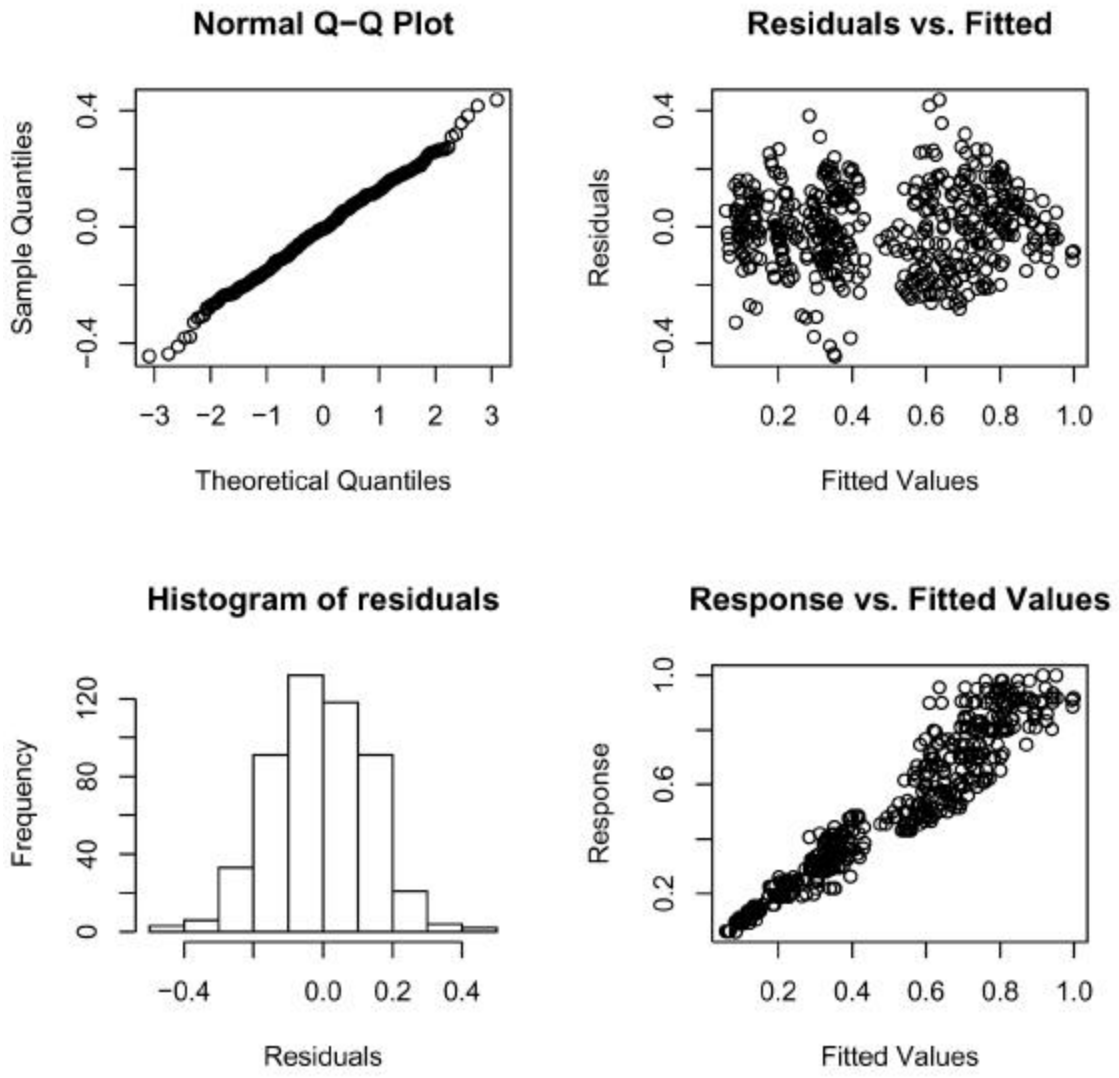

Figure 43. Diagnostic plot for Model 18, which used developmental stage, strain, temperature, length, weight, binary gene expression for four genes, and quantitative gene expression for nine genes, to predict *L. sericata* development percent (on a scale of 0–1). A gamma distribution was used with this model. Descriptions for each panel type can be found in Figure 4 of Generalized Additive Models. Predicted (Fitted) values represent a range of true (Response) values. The increase in error with age has diminished compared to other models and the gap between predictions for postfeeding third instar and pupal ages has shrunk.

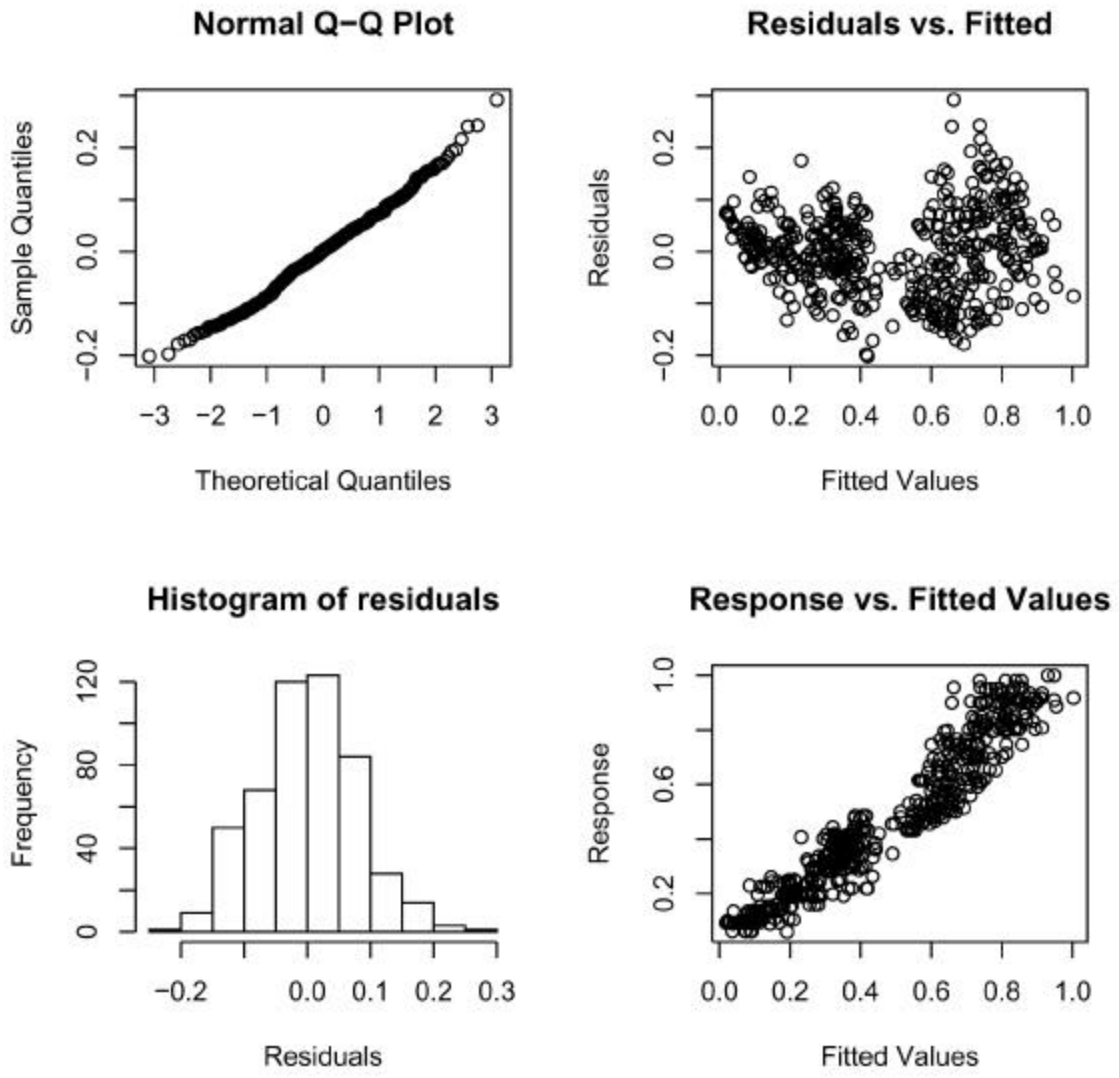

Figure 44. Diagnostic plot for Model 19, which used the same parameters as Model 18, to predict *L. sericata* development percent (on a scale of 0–1), but a gaussian distribution was used. Descriptions for each panel type can be found in Figure 4 of Generalized Additive Models. Predicted (Fitted) values represent a range of true (Response) values. The increase in error with age has diminished compared to Model 15 and the gap between predictions for postfeeding third instar and pupal ages has shrunk. However, compared to Model 18, this model exhibits more error in predictions of younger individuals.

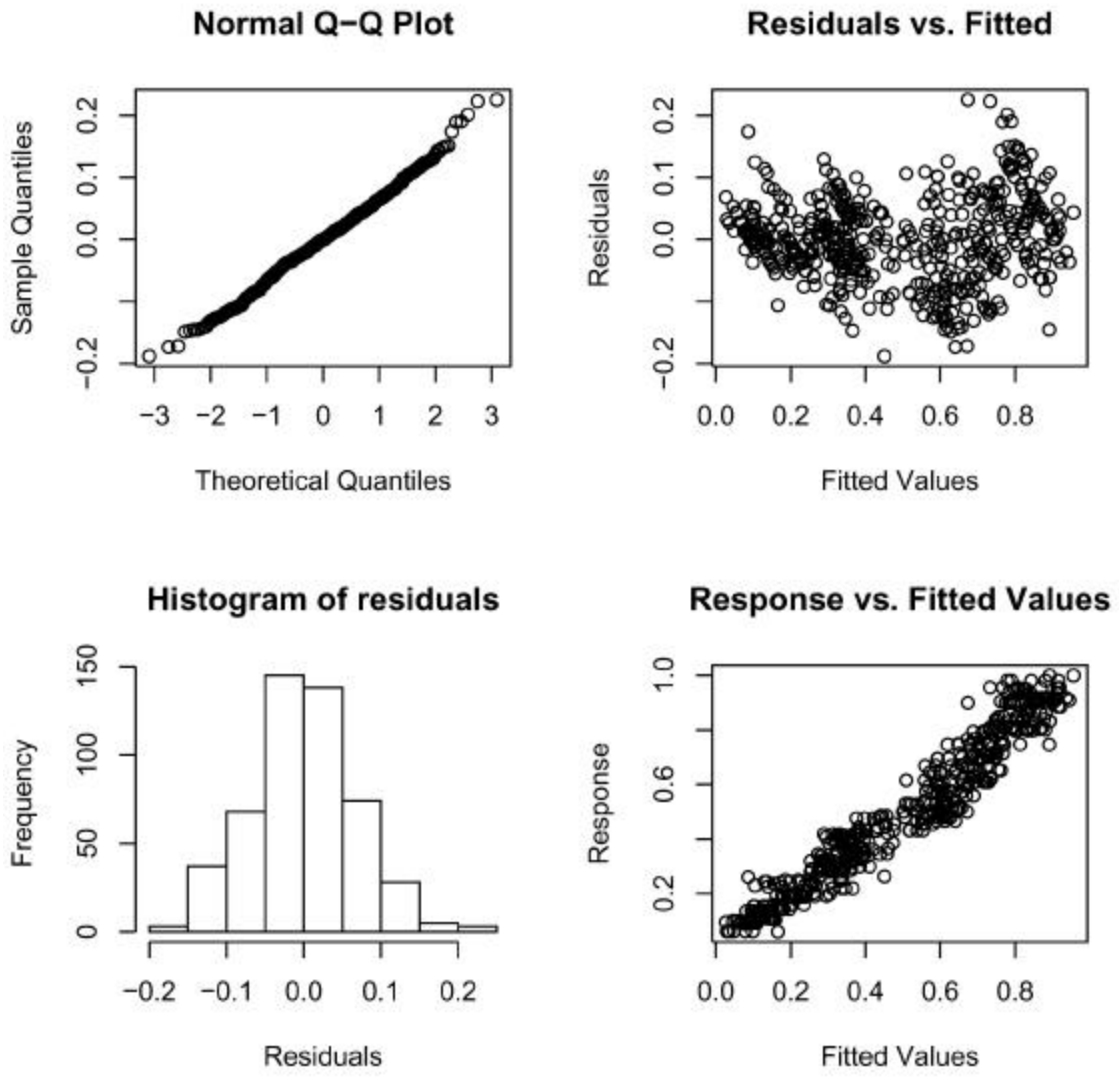

Figure 45. Diagnostic plot for Model 20, which used the same parameters as Model 18, to predict *L. sericata* development percent (on a scale of 0–1), but a gaussian distribution was used with this model and all genes expression scores were anchored against *hsp60* expression. Descriptions for each panel type can be found in Figure 4 of Generalized Additive Models. Predicted (Fitted) values represent a range of true (Response) values. The increase in error with age has diminished compared to Model 15 and the gap between predictions for postfeeding third instar and pupal ages has been eliminated. However, compared to Model 18, this model exhibits more error in predictions for younger individuals, though it is an improvement over predictions made with Model 19.

The last two models were similar to Model 21, but strain, temperature, and non-significant terms were removed. When compared to the models that preceded them, there was little change in the diagnostic plots (Figure 46–47 and Figures 47–48). Likewise, the statistical evaluations of the models revealed little change in the overall performance of the models compared to the model that preceded them. Model 22 had PDE and GCV scores of 94.6% and 0.0059, which represented no change in PDE and a GCV increase of 0.0003 compared to Model 21. Likewise, the removal of temperature (Model 23 compared to Model 22) resulted in PDE and GCV scores of 94.7% and 0.0059 for Model 23, which represented a 0.1% increase in PDE and no change in GCV compared to Model 22.

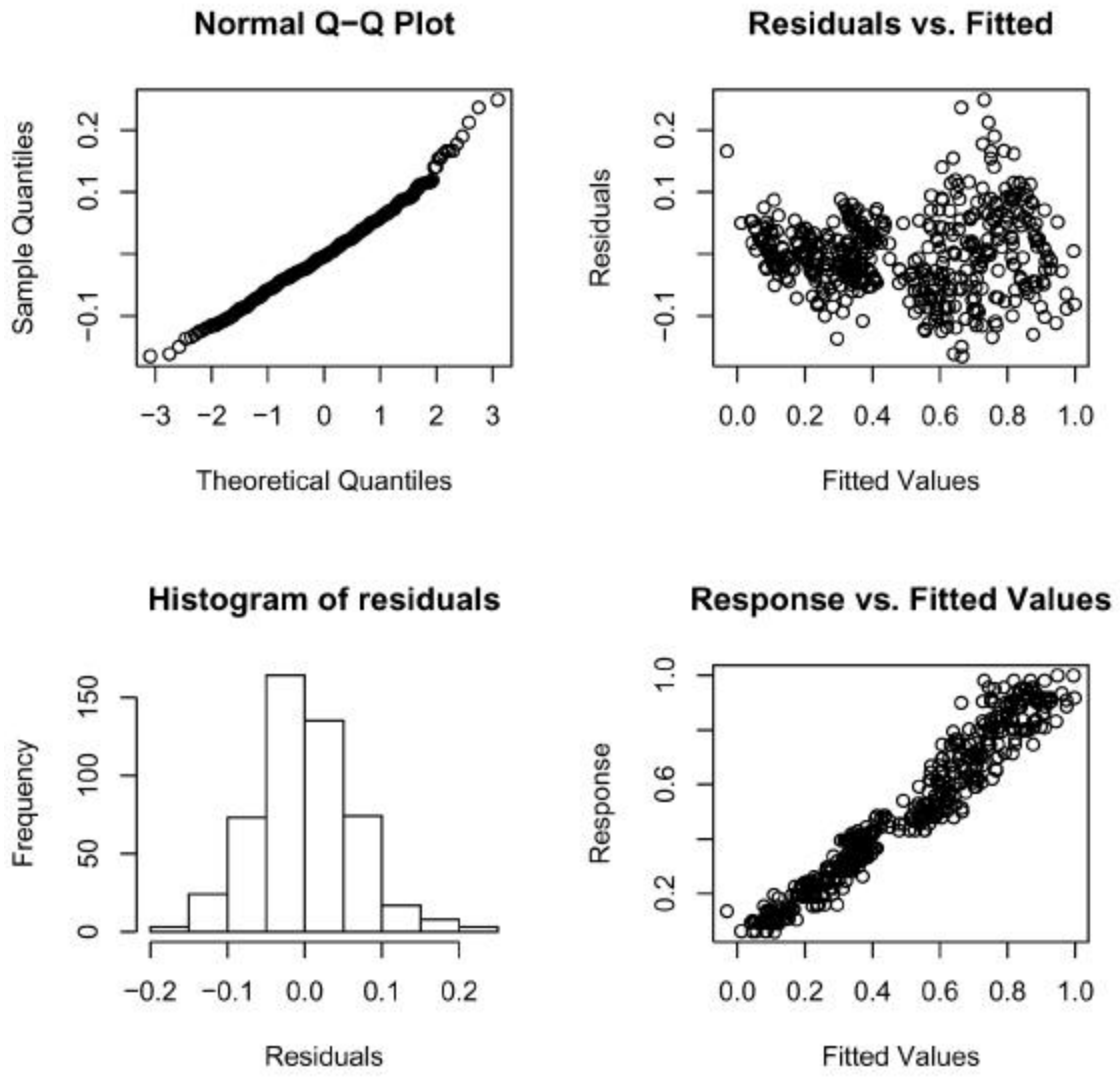

Figure 46. Diagnostic plot for Model 21, which used the same parameters as Model 18, to predict *L. sericata* development percent (on a scale of 0–1), but a gaussian distribution was used with this model and all genes expression scores were anchored against length measurements. Descriptions for each panel type can be found in Figure 4 of Generalized Additive Models. Predicted (Fitted) values represent a range of true (Response) values. The increase in error with age has diminished compared to Model 15 and the gap between predictions for postfeeding third instar and pupal ages has been eliminated. However, compared to Model 18, this model exhibits more error in predictions for younger individuals, though it is an improvement over predictions made with Model 19.

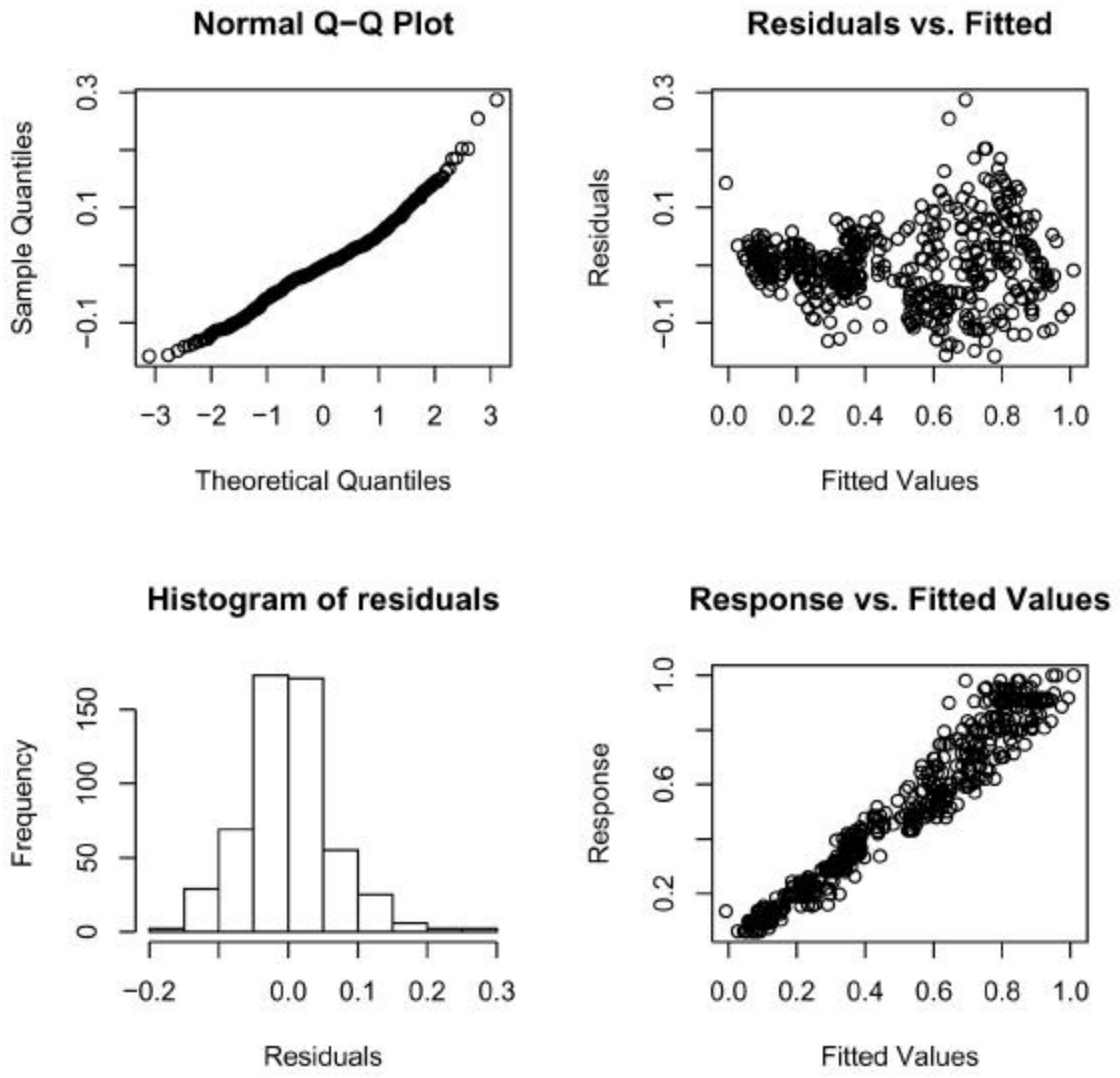

Figure 47. Diagnostic plot for Model 22, which used the same parameters as Model 21, to predict *L. sericata* development percent (on a scale of 0–1), but parameters that were non-significant predictors of age in Model 21 (length, weight, *cs*, *w*, strain) were removed. Descriptions for each panel type can be found in Figure 4 of Generalized Additive Models. Predicted (Fitted) values represent a range of true (Response) values. The increase in error with age has diminished compared to Model 15 and the gap between predictions for postfeeding third instar and pupal ages has been eliminated. However, compared to Model 18 this model exhibits more error in predictions for younger individuals, though it is an improvement over predictions made with Model 19. Removing strain, *cs*, and *w*, had little effect on the predictions made with the model compared to predictions made with Model 21.

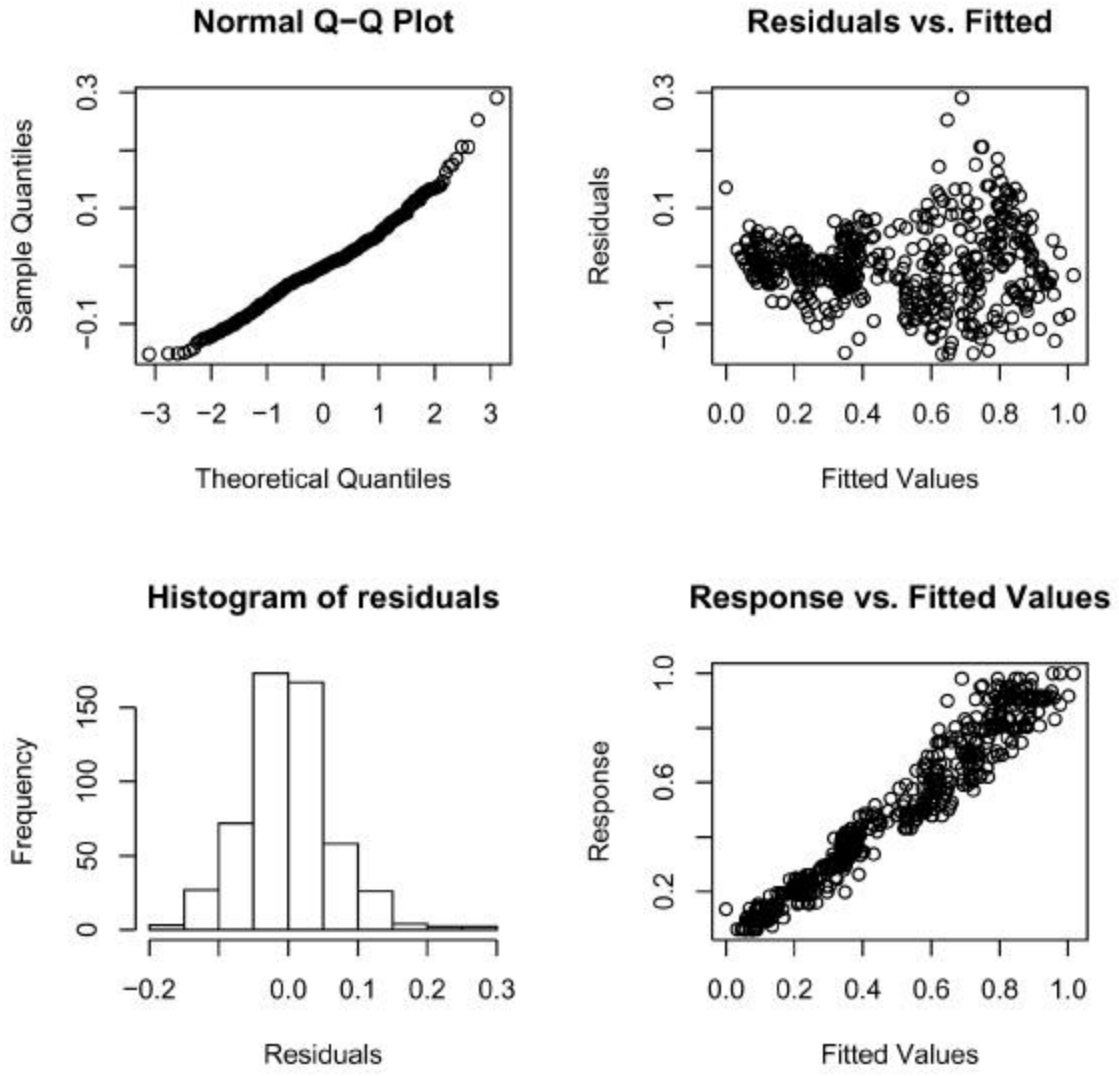

Figure 48. Diagnostic plot for Model 23, which used the same parameters as Model 22, to predict *L. sericata* development percent (on a scale of 0–1), except that temperature was removed from this model. Descriptions for each panel type can be found in Figure 4 of Generalized Additive Models. Predicted (Fitted) values represent a range of true (Response) values. Removing temperature had little effect on the predictions made with this model compared to predictions made with Model 22.

Finally, the differences between GAMS with or without gene expression data were examined for the most difficult to age stages, the postfeeding third instar and pupation. Here the influence of expression data was most vivid. As mentioned, there is very little age information

to be gained during the latter stages of development, as the animal has ceased changing in size. This leaves large errors in age estimates using conventional aging techniques (including stage/length/weight/temp/strain), with PDE values plummeting to 36.2% for third instars (Figure 49) and 15.8% for pupae (Figure 50). When gene expression data are included, these jump to 79.8% (Figure 51) and 78.2% (Figure 52) respectively.

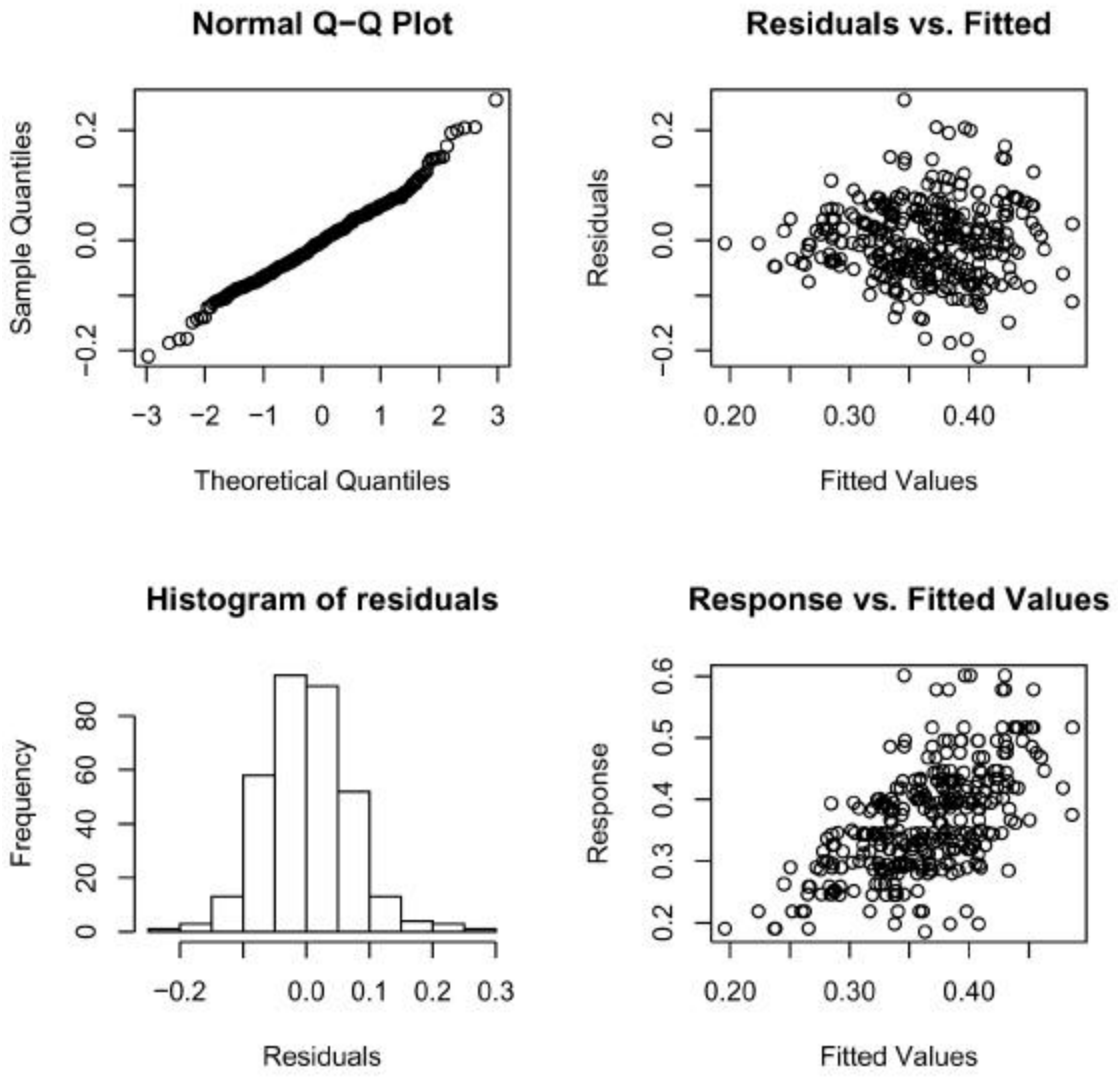

Figure 49. Diagnostic plot for postfeeding third instar incorporating stage/length/weight/temp/strain. Descriptions for each panel type can be found in Figure 4 of Generalized Additive Models. Note the extensive scatter in response vs. fitted values.

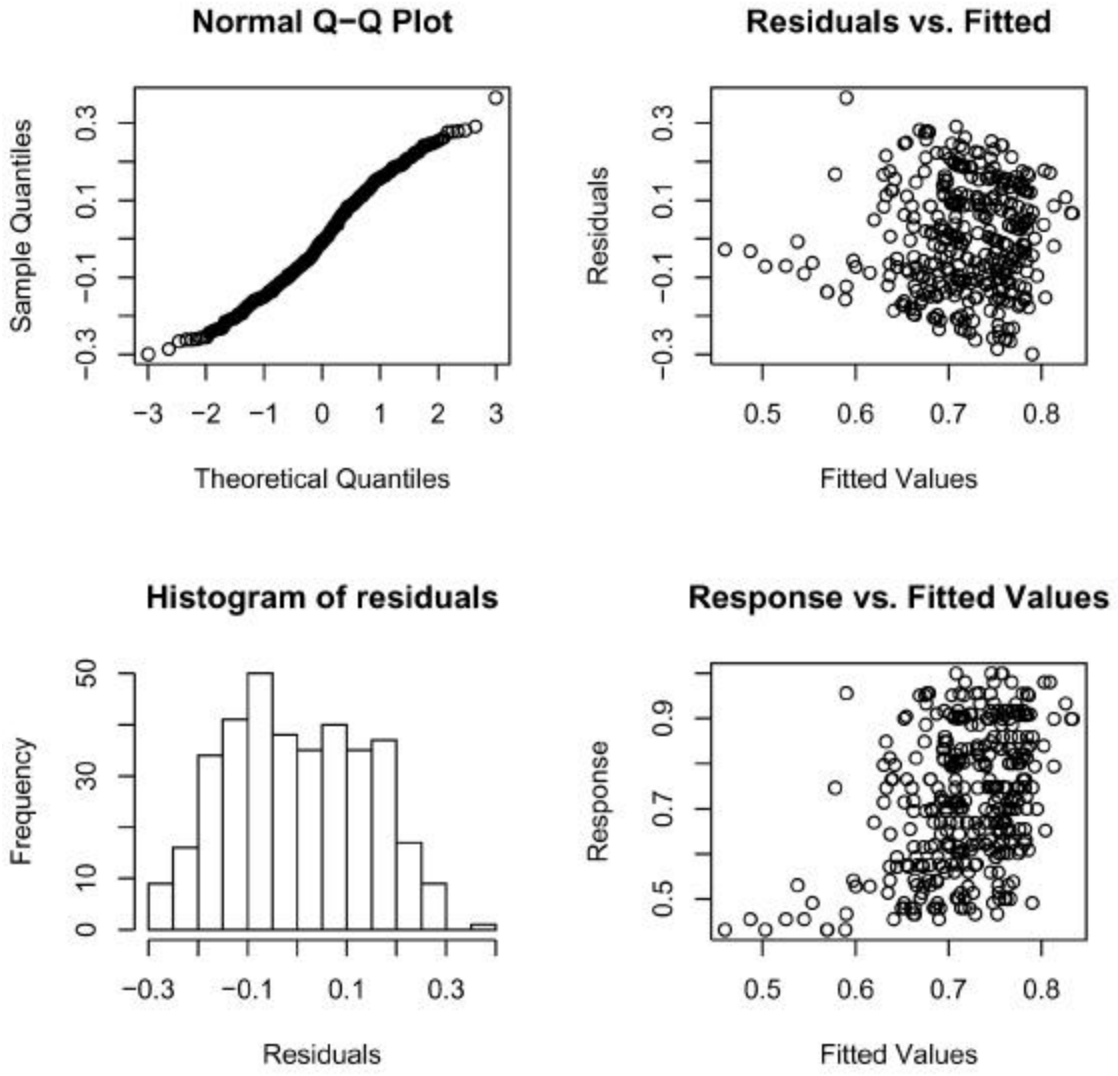

Figure 50. Diagnostic plot for pupae incorporating stage/length/weight/ temp/strain. Descriptions for each panel type can be found in Figure 4 of Generalized Additive Models. Note the extensive scatter in response vs. fitted values.

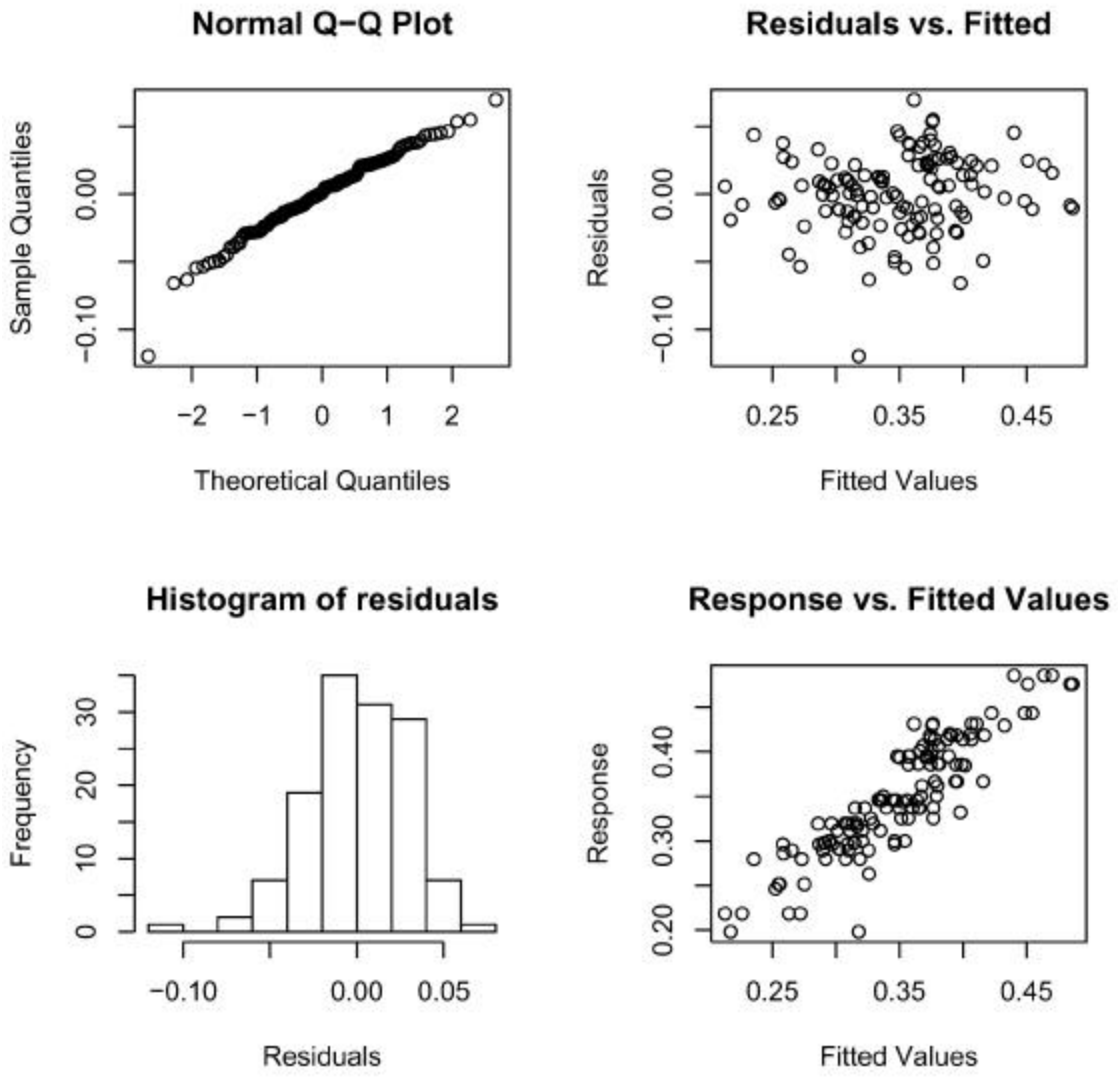

Figure 51. Diagnostic plot for postfeeding third instar incorporating stage/length/weight/ temp/strain and expression data. Descriptions for each panel type can be found in Figure 4 of Generalized Additive Models. Note the tightening in response vs. fitted values over Figure 49.

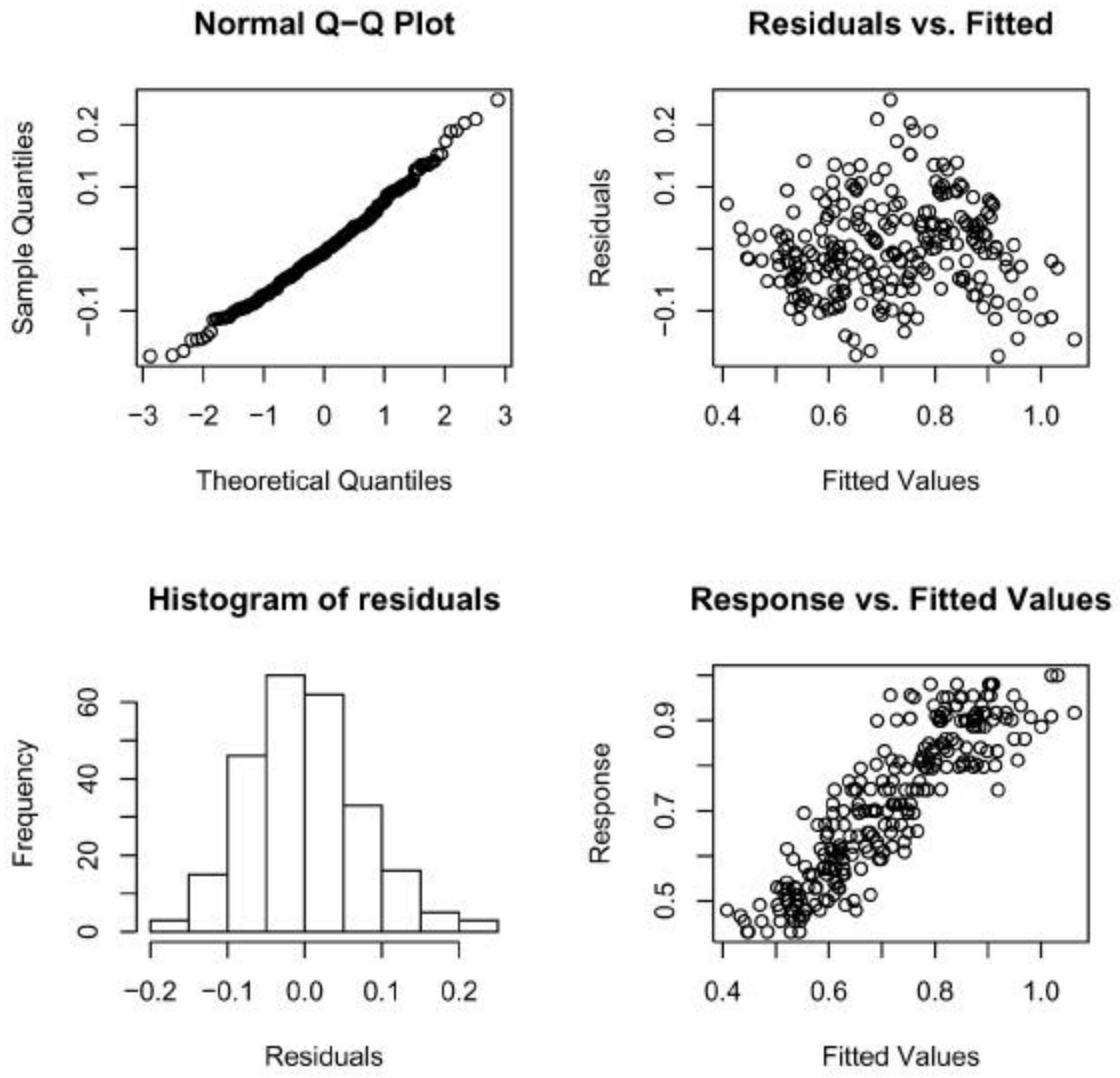

Figure 52. Diagnostic plot for postfeeding third instar incorporating stage/length/weight/ temp/strain and expression data. Descriptions for each panel type can be found in Figure 4 of Generalized Additive Models. Note the tightening in response vs. fitted values over Figure 50.

Discussion

Two important conclusions can be drawn from the research presented in this section. The first, and most important, is that the hypothesis that gene expression data can improve predictions of blow fly age was mathematically and graphically demonstrated (Tables 3, 4, and

Figures 41–52). The second is that the inclusion of strain and temperature into a GAM used to predict development percents of immature *L. sericata* had little effect on the outcome of predictions. Accordingly, it is reasonable to expect that the technique can be applied to different populations, raised at variable temperatures, with little overall effect on the results of a prediction.

The effective use of genetic data was most impressive during the third instar and pupation. This was anticipated, as it is these stages that are the longest, and are where only broad estimates of fly age can generally be made. The first two instars are typically about a day in length (temperature dependent) thus knowing developmental stage alone is an excellent predictor of age, and is where most of the accuracy in knowing just stage originates. Size is helpful during the feeding portion of the third instar, however once the larvae cease growing, accuracy tumbles. This is not really reflected in the general GAMs, as they look across development, not at specific parts of development. Once this was done however, the utility of gene expression data became obvious. Relatively little was learned from the standard traits in postfeeding third instar larvae, with only about 36% of the deviance in the data being explained. This, of course, stem form the body size change (shrinkage) that occurs once feeding ceases. During pupation, where size changes little or not at all, the PDE dropped to 16%. Addition of gene expression data made both of these values jump (80% and 78% respectively). It is where standard aging techniques are at their worst, where the genetic data were strongest.

In addition to increasing the precision of blow fly age predictions, the methodology is favorable to the standard approach for other reasons. First, the use of GAMs and their related statistics allows for a detailed understanding and description of error, an important part of the Daubert requirements for scientific evidence (see the Generalized Additive Models section for

more on this point). Second, almost any forensic laboratory that is qualified to work with DNA can conduct gene expression analyses, assuming they are using quantitative PCR, which many laboratories are. Finally, with robotics and microarrays (Arbeitman et al. 2002, Beckstead et al. 2006) the technique can be automated and miniaturized. All of these qualities make the analysis of gene expression data a powerful future tool for forensic entomological PMI predictions.

Table 1. Primers used for sequencing and qPCR for all loci in this experiment.

Primer	Function	Sequence	Template	Concentration (nM)
Tub R1	Sequencing	CACCAGATCGTTCATGTTGC	NM_166357	1000
Tub F1	Sequencing	CGAGACCTACTGCATCGACA	NM_166357	1000
qTub R	Quantitative PCR	ACCAGGCATGAAAAAGTGAAGAC	EF056211	400
qTub F	Quantitative PCR	TCCGTAAATTGGCCGTCAAC	EF056211	400
qRp49 F	Quantitative PCR	ACAATGTTAAGGAACTCGAAGTTTTG	AB118976	400
qRp49 R	Quantitative PCR	GGAGACACCGTGAGCGATTT	AB118976	400
ChS F2	Sequencing	GAACTGCCTATACCCGTGGA	AF221067	1000
ChS R2	Sequencing	GGATGTAAACACGCCGCTAT	AF221067	1000
qChS F	Quantitative PCR	GCCGACGGAGAACCTATACCA	EF056212	66.67
qChS R	Quantitative PCR	GATGGCTGTCATTGTGGGTACA	EF056212	400
qHsp60 F	Quantitative PCR	CATCATTCCCGCCCTTGA	AB118971	66.67
qHsp60 R	Quantitative PCR	ATCTTCGGCAATAATGACCAAAG	AB118971	400
qHsp90 F	Quantitative PCR	AAGATCATTTGGCTGTCAAGCA	AB118970	400
qHsp90 R	Quantitative PCR	AGAAGGGCACGGAATTCAAGT	AB118970	400
AcE F4	Sequencing	TATATGGGCTCCAGCAAAGG	U88631	1000
AcE R4	Sequencing	ATGGTACCCGATTGCATCAT	U88631	1000
qAcE F	Quantitative PCR	CACCGGTTATGCCAGGTTTT	NA	66.67
qAcE R	Quantitative PCR	TGATCCCAAAGGCCAACATT	NA	400
EcR F4	Sequencing	TTTCACCCTCGAGCAGTCTT	U75355	1000
EcR R3	Sequencing	CTTTCTTTTCGCGTCGTTTC	U75355	1000
qEcR F	Quantitative PCR	GCATGCGGCCGGAAT	NA	400
qEcR R	Quantitative PCR	GCGTCGTTTCATTGCACACT	NA	400
Usp F1	Sequencing	CGCAGGAGATAAAGCCAGAC	AY007213	1000
Usp R1	Sequencing	TGGTGTCGACGTGCATATT	AY007213	1000
qUsp F	Quantitative PCR	CGAGCAAAAAGCCGAATCAC	NA	400
qUsp R	Quantitative PCR	TGCCTACGCGCAAAAAGG	NA	400
qRop F	Quantitative PCR	GCCCCACTGTTGAGCCATA	AY691501	400
qRop R	Quantitative PCR	CCCGAGGATGTTTGGGTAAGA	AY691501	400
W F1	Sequencing	ACCGATCCTCCGCTCTTAAT	U38899	1000
W R1	Sequencing	TGATATCCAAGAACGCCACA	U38899	1000
qW F	Quantitative PCR	ACAACAGCCAAGACTTGGACATAG	NA	400
qW R	Quantitative PCR	GCGCCCAGTGTCCTACCA	NA	400
qSll F	Quantitative PCR	TCCAACGGCCACAATCTTAAGTA	AY926574	66.67
qSll R	Quantitative PCR	CGTTTAGGTGTTGCCGCAAT	AY926574	400
qWg F	Quantitative PCR	TGTCTGGTTCCTGTACGGTGAA	AY926575	400
qWg R	Quantitative PCR	TTATCGCCAATAACACGGAAATT	AY926575	66.67
Scl F4	Sequencing	CGCCATTGTGAACGTGATAC	U58977	1000
Scl R3	Sequencing	GCGAAAGCCAAAACTACGAG	U58977	1000
qScl F	Quantitative PCR	CGGAAGCGGCAGATTTTT	NA	400
qScl R	Quantitative PCR	TTCTCCGGGATTGGTGACA	NA	400
Rh3 F2	Sequencing	CGGCAAATCCTTATCGAAAT	AJ878411	1000
Rh3 R3	Sequencing	ACAAAACGTCCCCAACTTTC	AJ878411	1000
qRh3 F	Quantitative PCR	ACTACGAATGCTTTTATTGCCTTATG	NA	66.67
qRh3 R	Quantitative PCR	GCTTTGCCAGTGAGTCATTTTACC	NA	400

Quantitative PCR and sequencing primers used in these experiments. Tub= *ß Tubulin 56 D*. Rp49=*rp49*. ChS=*chitin synthase*. Hsp60=*heat shock protein 60*. Hsp90=*heat shock protein 90*. AcE=*acetylcholine esterase*. EcR=*ecdysone receptor*. Usp=*ultraspiracle*. Rop=*resistance to organophosphate* 1. W=*white*. Sll=slalom. Wg=wingless. Scl=*scalloped wings*. Rh3=*rhodopsin 3*. Template refers to accession numbers of the sequences used to construct the primers. NA in the template column indicates sequences that were obtained by this research, that are not yet submitted to NCBI. Concentration refers to the end concentration, in nM, of primers in a PCR reaction.

Table 2. Gene sequencing results.

Gene	Species	Size	Percent
ß tubulin 56 D	*Glossina morsitans morsitans*	635	86
chitin synthase	*Lucilia cuprina*	713	98
acetylcholine esterase	*Lucilia cuprina*	369	99
ecdysone receptor	*Lucilia cuprina*	350	98
	Lucilia sericata	102	100
ultraspiracle	*Lucilia cuprina*	683	95
	Lucilia sericata	508	99
white	*Lucilia cuprina*	861	95
scalloped wings	*Lucilia cuprina*	884	96
rhodopsin 3	*Calliphora vicina*	311	85

Species indicates the dipteran species that best matched a sequence. Size indicates the length in base pairs of match results for the sequence BLAST on the NCBI website. Percent indicates the percent similarity of that BLAST comparison.

Table 3. Generalized additive models assessed in this experiment.

Model	Model Parameters [Distribution (link)]	GCV	PDE	N
1	Percent = Stage [gamma(log)]	0.049	88.2	958
2	Percent = bin*cs*+Stage [gamma(log)]	0.049	88.5	958
3	Percent = bin*ace* [gamma(log)]	0.33	3.6	958
4	Percent = bin*w* [gamma(log)]	0.35	0.43	958
5	Percent = bin*sll*+Stage+Strain+Temp [gamma(log)]	0.048	88.5	958
6	Percent = s(*cs*)+Stage [gamma(log)]	0.046	89.5	856
7	Percent = s(*hsp60*) [gamma(log)]	0.41	14.7	852
8	Percent = s(*hsp90*)+Temp+Stage [gamma(log)]	0.048	88.8	947
9	Percent = s(*ace*)+Temp+Stage [gamma(log)]	0.046	89.9	636
10	Percent = s(*ecr*) [gamma(log)]	0.33	6.65	941
11	Percent = s(*rop-1*)+Stage [gamma(log)]	0.046	88.2	859
12	Percent = s(*w*) [gamma(log)]	0.31	3.55	725
13	Percent = s(*usp*)+Stage [gamma(log)]	0.047	88.8	849
14	Percent = s(*sll*) [gamma(log)]	0.34	9.17	677
15	Percent = Stage +Strain + Temp+ s(Length)+s(Weight)+s(Length,Weight) [gamma(log)]	0.038	91.8	958
16	Percent= Stage+Strain+Temp+s(L)+s(W)+s(L,W)+bin[caws] [gamma(log)]	0.037	92.1	958
17	Percent= Stage+Strain+Temp+s(L)+s(W)+s(L,W)+s(genes) [gamma(log)]	0.024	95.1	501
18	Percent= Stage+Strain+Temp+s(L)+s(W)+s(L,W)+s(genes) + bin[caws][gamma(log)]	0.024	95.1	501
19	Percent= Stage+Strain+Temp+s(L)+s(W)+s(L,W)+s(genes) + bin[caws] [gaussian(identity)]	0.0079	91.3	501
20	Percent= Stage+Strain+Temp+s(L)+s(W)+s(L,W)+s(*hsp60*,genes) + bin[caws] [gaussian(identity)]	0.007	93.7	501
21	Percent= Stage+Strain+Temp+s(L)+s(W)+s(L,W)+s(Length,genes) + bin[caws] [gaussian(identity)]	0.0056	94.6	501
22	Percent= Stage+Temp+s(L,W)+s(Length,genes) + bin[caws] [gaussian(identity)] no *cs* or *w*	0.0059	94.6	534
23	Percent= Stage+s(L,W)+s(Length,genes) + bin[caws] [gaussian(identity)] no *cs* or *w*	0.0059	94.7	534

The various models used to predict minimum development percent (Percent), the distributions applied to each model (gamma or gaussian), and the link function that applied the distribution to the model (identity or log). bin*locus* indicates the binary data for that gene. bin[caws] means all four significant binary variables (*cs*, *ace*, *w*, and *sll*) were included. s(*locus*) indicates a smoothing curve for that gene was analyzed. s(L) or s(W) indicates smoothing curves for length or weight were analyzed. s(variable,variable) indicates that a smoothed contour surface composed of the two variables was used to predict development percent. GCV is the generalized cross validation score for that model. PDE is the percent deviance explained by a model. N is the number of individuals included in that model.

Table 4. Estimated significance and degrees of freedom for terms in all GAMs.

Model	Variable	Linear Term	chi-squared	df/edf	P-value
1	Stage	Y	8816.2	4	<0.0001
2	Stage	Y	9031.3	4	<0.0001
	binary *chitin synthase*	Y	21.087	1	<0.0001
3	binary *acetylcholine esterase*	Y	46.911	1	<0.0001
4	binary *white*	Y	5.209	1	0.023
5	Stage	Y	9025.6	4	<0.0001
	Strain	Y	4.2436	2	0.12
	Temp	Y	22.828	1	<0.0001
	binary *slalom*	Y	8.0417	1	0.0047
6	Stage	Y	8797	4	<0.0001
	s(*chitin synthase*)	N	23.093	3.99	0.00014
7	s(*heat shock protein 60*)	N	156.36	6.96	<0.0001
8	Stage	Y	7926.3	4	<0.0001
	Temp	Y	28.806	1	<0.0001
	s(*heat shock protein 90*)	N	25.187	6.52	0.00054
9	Stage	Y	6502.8	4	<0.0001
	Temp	Y	12.568	1	0.00042
	s(*acetylcholine esterase*)	N	30.128	7.17	0.00013
10	s(*ecdysone receptor*)	N	82.179	5.46	<0.0001
11	Stage	Y	7309.2	4	<0.0001
	s(*resistance to organophosphate 1*)	N	62.44	5.47	<0.0001
12	s(*white*)	N	28.493	6.15	0.0001
13	Stage	Y	8167.2	4	<0.0001
	s(*ultraspiracle*)	N	27.006	3.58	<0.0001
14	s(*slalom*)	N	83.34	5.85	<0.0001
15	Stage	Y	483.29	4	<0.0001
	Strain	Y	23.608	2	<0.0001
	Temp	Y	94.234	1	<0.0001
	s(Length)	N	39.327	7.85	0.11
	s(Weight)	N	13.253	8.02	<0.0001
	s(Length,Weight)	N	68.628	15.2	<0.0001
16	Stage	Y	460.93	4	<0.0001
	Strain	Y	16.914	2	<0.0001
	Temp	Y	115.13	1	<0.0001
	s(Length)	N	21.342	7.13	0.0039
	s(Weight)	N	15.804	4.91	0.0073
	s(Length,Weight)	N	80.472	21.9	<0.0001
	binary *chitin synthase*	Y	32.382	1	<0.0001
	binary *acetylcholine esterase*	Y	0.1385	1	0.71
	binary *white*	Y	0.73592	1	0.39
	binary *slalom*	Y	0.073636	1	0.79
17	Stage	Y	232.56	4	<0.0001
	Strain	Y	3.9962	2	0.14

	Temp	Y	62.513	1	<0.0001
	s(Length)	N	30.504	5.61	<0.0001
	s(Weight)	N	8.6437	1.27	0.0054
	s(Length,Weight)	N	37.219	11.8	0.00028
	s(*chitin synthase*)	N	8.7356	2.98	0.034
	s(*heat shock protein 60*)	N	52.67	5.75	<0.0001
	s(*heat shock protein 90*)	N	16.536	1.92	0.00027
	s(*acetylcholine esterase*)	N	80.381	7	<0.0001
	s(*ecdysone receptor*)	N	21.546	1	<0.0001
	s(*resistance to organophosphate 1*)	N	44.075	5.07	<0.0001
	s(*white*)	N	2.9499	1.63	0.17
	s(*ultraspiracle*)	N	23.967	5.25	<0.0001
	s(*slalom*)	N	1.5654	1	0.21
18	Stage	Y	232.56	4	<0.0001
	Strain	Y	3.9962	2	0.17
	Temp	Y	62.513	1	<0.0001
	s(Length)	N	30.504	5.61	<0.0001
	s(Weight)	N	8.6437	1.27	0.0054
	s(Length,Weight)	N	37.219	11.8	0.00028
	s(*chitin synthase*)	N	8.7356	2.98	0.034
	s(*heat shock protein 60*)	N	52.67	5.75	<0.0001
	s(*heat shock protein 90*)	N	16.536	1.92	0.00027
	s(*acetylcholine esterase*)	N	80.381	7	<0.0001
	s(*ecdysone receptor*)	N	21.546	1	<0.0001
	s(*resistance to organophosphate 1*)	N	44.075	5.07	<0.0001
	s(*white*)	N	2.9499	1.63	0.17
	s(*ultraspiracle*)	N	23.967	5.25	0.00035
	s(*slalom*)	N	1.5654	1	0.21
	binary *chitin synthase*	Y	101.58	1	<0.0001
	binary *acetylcholine esterase*	Y	101.58	1	<0.0001
	binary *white*	Y	101.58	1	<0.0001
	binary *slalom*	Y	101.58	1	<0.0001
19	Stage	Y	107.61	4	<0.0001
	Strain	Y	5.7489	2	0.057
	Temp	Y	22.098	1	<0.0001
	s(Length)	N	0.3813	1	0.54
	s(Weight)	N	4.9028	1	0.027
	s(Length,Weight)	N	51.222	14.5	<0.0001
	s(*chitin synthase*)	N	4.8273	1	0.029
	s(*heat shock protein 60*)	N	73.642	4.34	<0.0001
	s(*heat shock protein 90*)	N	24.305	3.64	<0.0001
	s(*acetylcholine esterase*)	N	89.688	6.41	<0.0001
	s(*ecdysone receptor*)	N	18.27	1	<0.0001
	s(*resistance to organophosphate 1*)	N	51.348	6.19	<0.0001
	s(*white*)	N	0.65226	1	0.42
	s(*ultraspiracle*)	N	35.758	3.86	<0.0001
	s(*slalom*)	N	2.1667	2.15	0.37

	binary *chitin synthase*	Y	23.006	1	<0.0001
	binary *acetylcholine esterase*	Y	23.006	1	<0.0001
	binary *white*	Y	23.006	1	<0.0001
	binary *slalom*	Y	23.006	1	<0.0001
20	Stage	Y	120.09	4	<0.0001
	Strain	Y	11.099	2	0.0042
	Temp	Y	10.942	1	0.001
	s(Length)	N	6.7474	3.38	0.11
	s(Weight)	N	4.8594	1.2	0.038
	s(Length,Weight)	N	24.463	9.38	0.0054
	s(*heat shock protein 60, chitin synthase*)	N	41.961	17.8	0.0015
	s(*heat shock protein 60, heat shock protein 90*)	N	39.926	7.75	<0.0001
	s(*heat shock protein 60, acetylcholine esterase*)	N	63.749	7.54	<0.0001
	s(*heat shock protein 60, ecdysone receptor*)	N	56.883	15	<0.0001
	s(*heat shock protein 60, resistance to organophosphate 1*)	N	58.023	15.4	<0.0001
	s(*heat shock protein 60, white*)	N	0.58248	1	0.46
	s(*heat shock protein 60, ultraspiracle*)	N	50.482	9.58	<0.0001
	s(*heat shock protein 60*, *slalom*)	N	0.29664	1.09	0.63
	binary *chitin synthase*	Y	18.171	1	<0.0001
	binary *acetylcholine esterase*	Y	18.171	1	<0.0001
	binary *white*	Y	18.171	1	<0.0001
	binary *slalom*	Y	18.171	1	<0.0001
21	Stage	Y	167.71	4	<0.0001
	Strain	Y	20.991	2	<0.0001
	Temp	Y	16.04	1	<0.0001
	s(Length)	N	0.79759	1	0.37
	s(Weight)	N	11.685	2.99	0.0091
	s(Length,Weight)	N	0.56452	0.37	0.21
	s(Length, *chitin synthase*)	N	2.7732	1	0.097
	s(Length, *heat shock protein 60*)	N	130.35	9.69	<0.0001
	s(Length, *heat shock protein 90*)	N	64.9	10.1	<0.0001
	s(Length, *acetylcholine esterase*)	N	103.54	14.2	<0.0001
	s(Length, *ecdysone receptor*)	N	38.517	1	<0.0001
	s(Length, *resistance to organophosphate 1*)	N	56.472	12	<0.0001
	s(Length, *white*)	N	1.1918	1	0.28
	s(Length, *ultraspiracle*)	N	107.65	14.9	<0.0001
	s(Length, *slalom*)	N	30.064	7.77	0.00024
	binary *chitin synthase*	Y	17.721	1	<0.0001
	binary *acetylcholine esterase*	Y	17.721	1	<0.0001
	binary *white*	Y	17.721	1	<0.0001
	binary *slalom*	Y	17.721	1	<0.0001
22	Stage	Y	207.64	4	<0.0001
	Temp	Y	12.111	1	0.00055
	s(Length,Weight)	N	15.495	5.48	0.013
	s(Length, *heat shock protein 60*)	N	143.36	11	<0.0001
	s(Length, *heat shock protein 90*)	N	45.018	8.54	<0.0001
	s(Length, *acetylcholine esterase*)	N	96.334	13.1	<0.0001

	s(Length, *ecdysone receptor*)	N	67.053	13.6	<0.0001
	s(Length, *resistance to organophosphate 1*)	N	81.328	10.5	<0.0001
	s(Length, *ultraspiracle*)	N	111.35	15.1	<0.0001
	s(Length, *slalom*)	N	20.687	6.9	0.0046
	binary *chitin synthase*	Y	5.1045	1	0.024
	binary *acetylcholine esterase*	Y	17	1	<0.0001
	binary *white*	Y	0.071709	1	0.79
	binary *slalom*	Y	17	1	<0.0001
23	Stage	Y	193.9	4	<0.0001
	s(Length,Weight)	N	18.197	5.39	0.0042
	s(Length, *heat shock protein 60*)	N	140.01	13.3	<0.0001
	s(Length, *heat shock protein 90*)	N	38.366	7.27	<0.0001
	s(Length, *acetylcholine esterase*)	N	104.41	15.4	<0.0001
	s(Length, *ecdysone receptor*)	N	74.27	14.8	<0.0001
	s(Length, *resistance to organophosphate 1*)	N	70.912	10.3	<0.0001
	s(Length, *ultraspiracle*)	N	142.34	16.4	<0.0001
	s(Length, *slalom*)	N	18.154	7.02	<0.0001
	binary *chitin synthase*	Y	4.0422	1	0.045
	binary *acetylcholine esterase*	Y	10.675	1	0.0012
	binary *white*	Y	0.023118	1	0.88
	binary *slalom*	Y	10.675	1	0.0012

The significance and degrees of freedom for all variables in all GAMs assessed in this project. For genetic data, the simplest model is shown in which a gene was statistically significant. Model 21 contains all available data; after that model, non-significant variables were removed. All terms were significant predictors of age in at least one model. Linear term indicates whether (Y) or not (N) a term is a linear variable. Df=degrees of freedom. edf=estimated degrees of freedom. P-value=estimated P-value. See the Generalized Additive Models section for greater details on models.

Validation with Blind Predictions

The results presented in the Larval and Pupal Gene Expression section indicated that predictions of blow fly development percent made with gene expression data are more precise than current forensic entomology approaches. However, as in Generalized Additive Models, the predicted performance of a model does not necessarily ensure that predictions will follow the expected pattern. To prove that a statistical model is a valuable predictor of development percent it is necessary to validate it (Scheiner and Gurevitch 2001), in this case by estimating the ages of unknown immature blow flies in a blind study. If the predicted ages of individuals plot along a True = Predicted line, then a model can be considered validated for use.

Methods

Strain Collection and Identification.

To avoid the effects of inbreeding resulting from over-winter rearing, a new collection of *L. sericata* was caught out of doors on the Michigan State University campus in May of 2006. Individuals were identified visually and by *CO1* sequence, as described in Generalized Additive Models.

Collection of Unknowns.

Egg masses were placed in the mouths of three CO_2 asphyxiated rat carcasses as described in Developmental Plasticity. Two cohorts were raised in incubators, one at 20ºC and one at 33.5ºC. Rearing conditions were the same as in Developmental Plasticity. The third cohort was raised in a sealed terrarium under outdoor ambient conditions. The terrarium was covered with a tightly fitting foam lid, which had a hole cut in the middle to allow air in. The hole was sealed by a screen, which kept other flies out of the terrarium.

Several individual larvae or pupae were collected daily from each of the three cohorts. An independent researcher recorded the ages of the individuals, which were stored in RNAlater (Applied Biosystems) at –80ºC. Pupae were collected as described in Generalized Additive Models for the stable temperature treatments; in the terrarium no attempt was made to separate pupae by age. Cohorts were checked daily until adults were observed, which was recorded as the minimum development time for the cohort; all development percents were calculated based on minimum development time.

For the ambient temperature experiment temperature variance was noted using a HOBO data logger (Onset Computer, Bourne, MA). Temperature data were used to calculate accumulated degree hours (ADH) by subtracting a base temperature of 10ºC from the recorded temperature at any hour and adding the hourly values together. This resulted in an accumulated degree hour (ADH) calculation. The total ADH until eclosion was used for the minimum development time and the ADH at the time of collection was divided by that number to calculate minimum development percent for the ambient temperature cohort.

Converting RNAlater Size Measurements to Live Size Measurements.

Since the body size measurements in the previous section were based on live *L. sericata,* and the unknown individuals were presented for analysis stored in RNAlater, it was necessary to develop a protocol for converting size measurements of individuals stored in solution to an estimate of live size. Unanalyzed individuals (N=400) from the MI collections in Generalized Additive Models were remeasured and reweighed after approximately one year of storage in RNAlater at –80ºC. The new measurements and weights were plotted against the recorded live data. Regression equations for the comparisons were used to convert the sizes of the unknown individuals to usable data for GAMs.

Molecular Biology and Statistics.

Gene expression CT scores were obtained as described in the previous section, which were used to predict the ages of blind study individuals. Several predictions were made for each individual, beginning with a GAM that included length, weight, and developmental stage to predict development percent. Likewise, GAMs were produced utilizing body size measurements, developmental stage, and all available gene expression information. Either the gamma or gaussian distributions were applied to the models, depending on which better satisfied the assumptions of error (discussed in Generalized Additive Models). Models were also developed with gene CT scores, in which each gene expression term was anchored [i.e. the term s(variable, gene) was used] against length measurements [s(length,gene)], as well as *hsp60* CT scores [s(*hsp60*,gene)] when *hsp60* was detected (see Table 3 in Larval and Pupal Gene Expression).

Two to four predictions were generated for each individual. Development percents were predicted for all individuals based on body size and developmental stage data. An additive model was also developed for each individual, using all available data. If an individual was raised at a known temperature, then temperature was included in the model. Strain was not included, as few investigators will have that information. If a diagnostic plot indicated that anchoring a prediction with length was not likely to improve upon other predictions made with body size and stage (based on the description of model types below), then a length anchored prediction was not used.

Predicted values of development percent were plotted against true development percent, and were visualized with the Y = X (True = Predicted) line ± 10%. A GAM was classified as a Type 1 model if its diagnostic plot was similar to Model 15 from the previous section, or as a

Type 2 model if the diagnostic plot indicated no gap in predictions and a decrease in the error throughout development. Predictions from Type 1 and Type 2 models were plotted and compared to predictions made with body size and stage information only.

Results

The new strain of Michigan *L. sericata* was identified by visual identification and through *CO1* sequence. A BLAST search on the NCBI website revealed that a 753 base pair fragment of the *CO1* gene was most similar to another *L. sericata* sequence, with 100% similarity. The closest match to non-*L. sericata* sequence was a 99% match to that gene in *L. cuprina*, with a 7 base pair mismatch.

Ambient temperatures spiked each day, then decreased to an evening low (Figure 1). Despite the fluctuations, the ADH based development percents were very similar to the purely temporal development percents (Figure 2). Because ADH is generally used in forensic entomology they were used for the analyses detailed below.

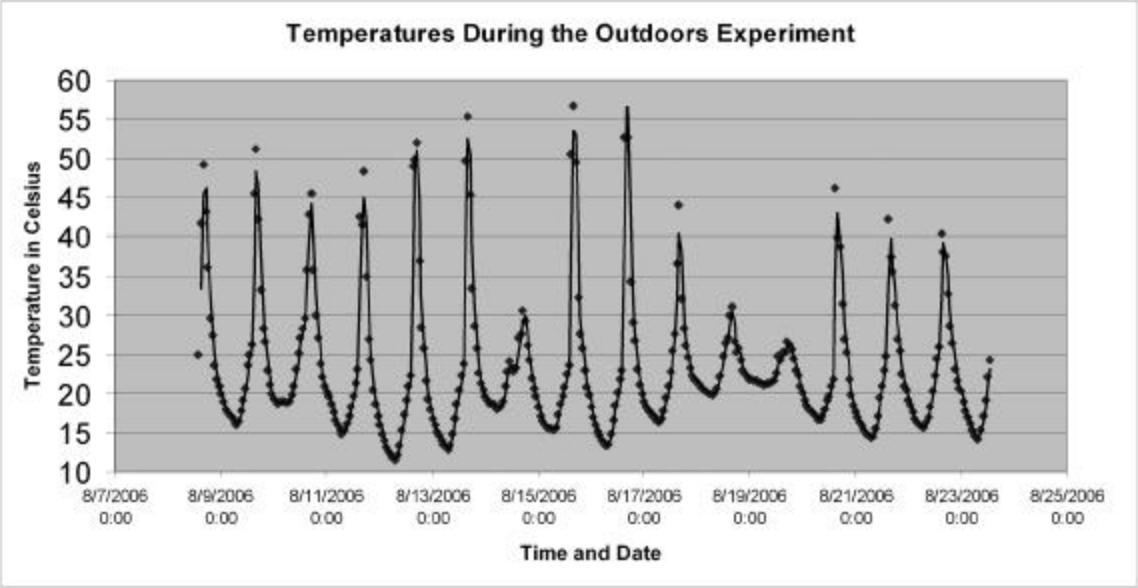

Figure 1. Temperature plots for the ambient temperature experiment. Temperatures spiked in the afternoon as sunlight was directly on the terrarium.

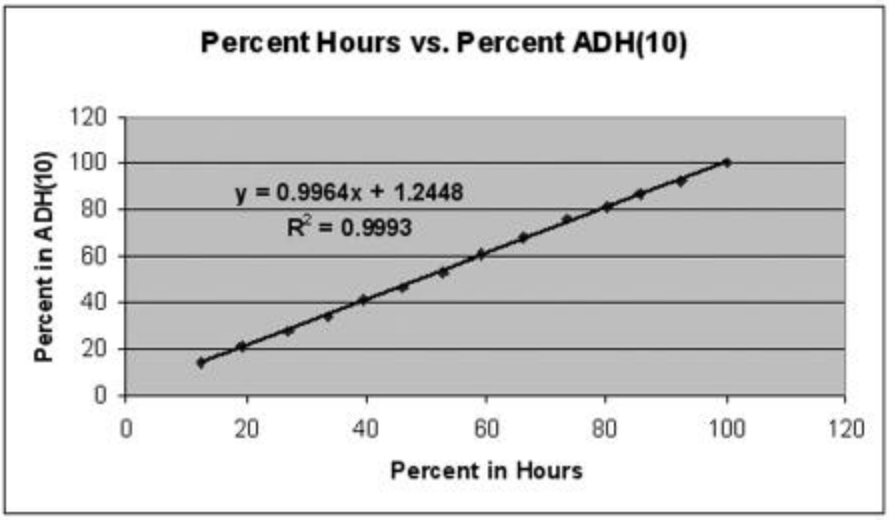

Figure 2. The regression of ADH (base 10) percents versus percent development in hours. The values were strongly correlated, with an R-squared and slope almost 1. However, the 1.2448 shift up the Y axis indicates that ADH is a better measure of developmental progress.

Ninety individuals were used to obtain cDNA samples for the blind study. Four of these were non-pupators (see the next section) and 86 were normally developing larvae and pupae.

Body size measurements were calculated based on the equations in Tables 1–2, which were specific to each stage. Seven flies were identified as first instars, 4 were second instars, 10 were feeding third instars, 23 were postfeeding third instars (based on crop content), and 42 were pupae. The 86 individuals were divided among temperature treatments with 34 raised at 20ºC, 29 raised at 33.5ºC, and 23 raised at ambient temperature. Of the postfeeding third instar identifications, 5 were retrospectively found to be feeding third instars, but predictions were made using the identified stage, as an investigator would not have been able to recognize a misidentified stage. Forty-nine individuals generated data that could be used in Type 2 unanchored models. Fifty-six individuals received *hsp60* anchored predictions. The ages of 71 flies were predicted based on models that used gene expression data anchored by length.

The plot for predicted versus true age for a GAM using developmental stage and body size to predict development percent (Figure 3) was used as the benchmark against which all other predictions were compared. The plot also included predictions for the four non-pupators (in the gray bar), which will be discussed in more detail in the next section. For the purposes of validation, the non-pupators were not considered further. If the hypothesized increase in precision occurred as predicted, then the results of plots from the models that used gene expression to predict blow fly age should demonstrate an improvement over the results in Figure 3. Additionally, the error associated with these models was considered. Based on the diagnostic plots of gene expression GAMs (from the previous section), approximately 90% of the data should be within 10% of the true age. With realistic collection conditions, individuals that are younger than the minimum age are expected (only a subset of flies on a body can be the oldest), thus underpredictions of age are anticipated. However, the minimum development time of a blow fly is the important information used by forensic entomologists, thus very little

overprediction of blow fly age should be tolerated in a model that will be used to predict this datum. When body size and stage data were used to predict blow fly age (Figure 3), the predictions of larval age was within 10% of true development except for 6 individuals. However, many pupal ages were predicted as greater than 10% older or 10% younger than their true ages. Likewise, few individuals were predicted to be between 40 and 60 percent developed, and no individuals were predicted to be greater than 75% developed.

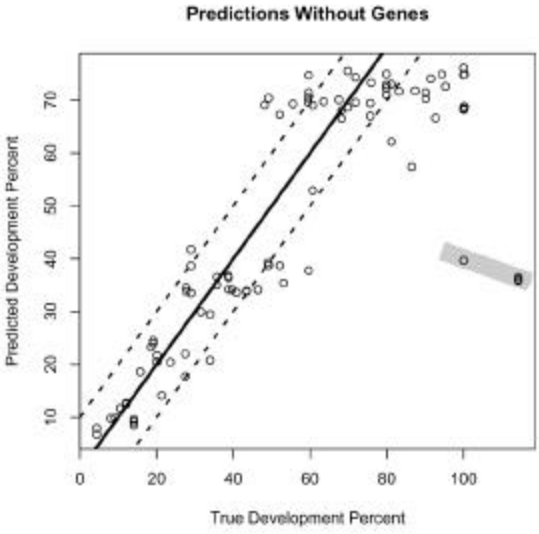

Figure 3. Predicted versus true development percents for all 90 individuals in the blind study. Predictions were made with GAMs that did not use gene expression information. The four points in the gray bar represent the four larvae that failed to pupate, and were eliminated from analyses in this section.

All gene expression models (Figures 4–10) improved upon the GAM predictions made with body size/stage data (Figure 3). When unanchored additive gene expression predictions were compared to their true ages (Figure 4), 100% of the larvae were predicted as within10% of their true ages or younger than their true age. Similarly, 85.7% of pupae were predicted as less than 10% older than their true development percents. Most importantly, pupal age predictions

were more precise than the size/stage predictions of age, as seen by the reduced size of the gap between larval and pupal age predictions. Additionally, development percents were accurately predicted when true percents were greater than 80%. When only the Type 2 additive model predictions (which included models predicting development percent with complete, or almost complete, gene expression profiles) were plotted against their true development percents (Figure 5) 93.9% of individuals were predicted as within or less than 10% of their true ages. In the three cases that larvae were predicted as older than 10% of true age, the predictions were only marginally greater than 10% deviant from the true development percent, which was not the case for predictions of individuals from the preceding models (Figures 3–4). Length anchored predictions followed a similar pattern, overall and for Type 2 models (Figure 6–7). Over-predicted development percents (>10%) with Type 2 models of this type only occurred twice (again, with less deviation from the true age than the size/stage based model). However, models that used *hsp60*-anchored predictions produced too many imprecise predictions to be considered useful and were not considered further.

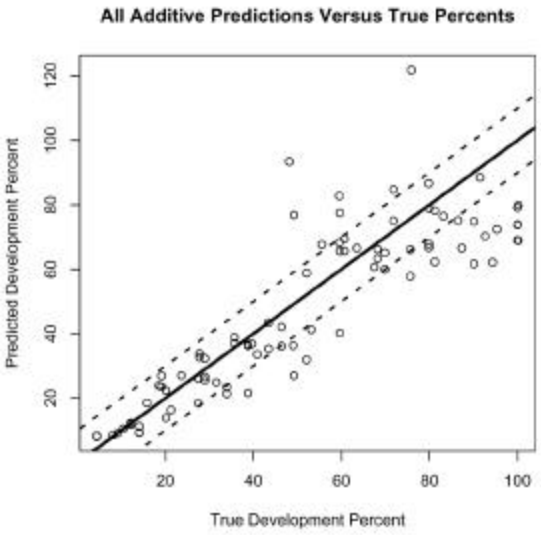

Figure 4. Predicted versus true development percents for the 86 normally developing individuals in the blind study. Predictions were made with GAMs that used unanchored gene expression terms.

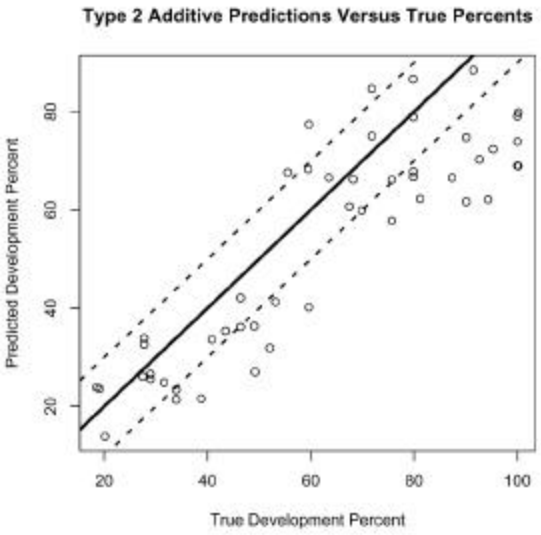

Figure 5. Predicted versus true development percents for 49 normally developing individuals in the blind study. Predictions were made with GAMs that used unanchored gene expression terms and were Type 2 models.

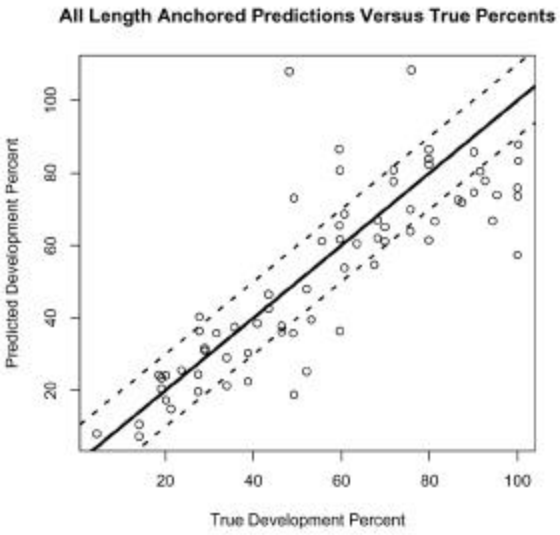

Figure 6. Predicted versus true development percents for 71 normally developing individuals in the blind study. Predictions were made with GAMs that used length anchored gene expression terms.

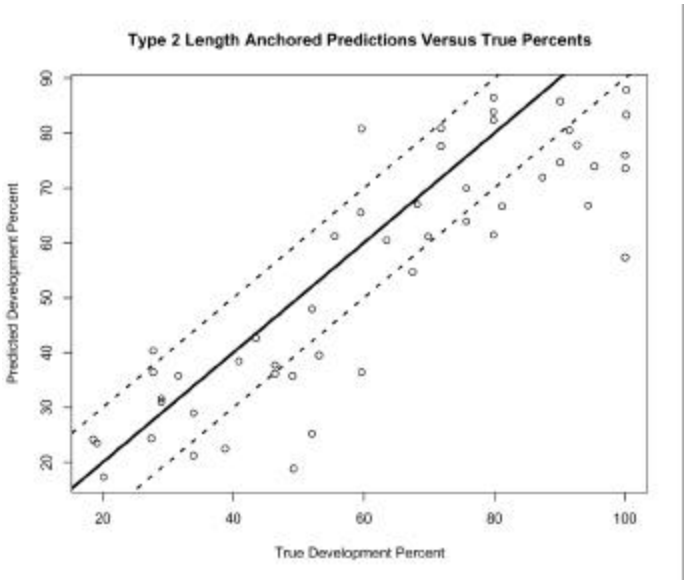

Figure 7. Predicted versus true development percents. Predictions were made with GAMs that used length anchored gene expression terms and were Type 2 models.

The performances of Type 2 additive models were also evaluated by temperature, but it was not possible to determine if certain temperatures were associated with more accurate

predictions than others; since 54.4% of profiles were predicted with unanchored additive Type 2 models and each temperature was only a third of the remaining 49 individuals there was little data to compare. The 20ºC predictions seemed the most accurate, but more research (with larger sample sizes) will be required to establish whether or not temperature (as well as other environmental factors) affect predictions made with such data.

All models continued to demonstrate a bias toward predicting individuals to be younger than their true ages. Adding gene expression information did not eliminate underestimates of age, however, the frequency of underestimates was less than those made with the models in Figure1.

Discussion

The results of the blind study demonstrated the utility of using gene expression for predicting blow fly age, and the use of GAMs to predict blow fly age by incorporating gene expression data was validated. When complete or nearly complete gene expression profiles (Type 2 models) were used to predict blow fly age, the predictions were much more precise. Both gene based GAMs and GAMs that used length anchored gene expression terms provided more precise predictions of age, generating far more blow fly age estimates within 10% of actual age. In doing so, a number of problems with the current PMI prediction process have been addressed. Currently, the range of development percents for pupal samples is ranges from ~40–100% (see the Generalized Additive Models section) if standard entomological measurements are employed. The use of gene expression clearly decreases this range. Likewise, the predictions produced with gene expression GAMs yielded a more seamless transition of age

estimates from postfeeding third instar to pupa. As well, estimates of ages greater that 10% above true age, which are the least desirable, were decreased remarkably with these models.

Error in age predictions calling individuals younger than they were, were more common. This phenomenon occurred independent of the use of gene expression, so it is not a signature shortcoming of using RNA profiles to predict age, and indeed, occurred less often with gene expression data, indicating that it was the standard data that resulted in this phenomenon. An explanation for underestimates lies in the results of the Developmental Plasticity chapter. *L. sericata* development is known to vary, and it is not likely that all collected individuals developed at the maximum rate, thus some individuals could truly be less mature than the most developed of their cohort. In natural conditions, underestimates of age will be likely; as females do not stop laying after the first female has oviposited on a corpse. However, the most important data from a blow fly age estimate is the predicted minimum development time/PMI. Given that there is a maximum development rate, as long as a model is not likely to predict individual ages that are older than their true age, then the minimum development time will be precisely estimated, as was the case in the blind study. Additionally, this problem can be largely overcome by sampling multiple individuals. If many individuals are sampled from a death scene, then the most developed can be used as an indicator of the minimum amount of time that flies could have been colonizing remains.

Table 1. Regressions of live length against preserved length for each stage.

Stage	Regression Equation	P-value	R^2
1st Instar	Length=1.062*RNA Later Length+0.3	<0.0001	0.76
2nd Instar	Length=1.548*RNA Later Length-0.48	<0.0001	0.77
3rd Instar (feeding)	Length=1.095*RNA Later Length+2.2	<0.0001	0.89
3rd Instar (postfeeding)	Length=0.7371*RNA Later Length+5.2	<0.0001	0.59
Pupa	Length=0.9059*RNA Later Length+0.65	<0.0001	0.88

Table 2. Regressions of live weight against preserved weight for each stage.

Stage	Regression Equation	P-value	R^2
1st Instar	Weight=1.078*RNA Later Weight+0.07	<0.0001	0.87
2nd Instar	Weight=1.077*RNA Later Weight-0.04	<0.0001	0.98
3rd Instar (feeding)	Weight=1.009*RNA Later Weight+0.12	<0.0001	0.99
3rd Instar (postfeeding)	Weight=1.047*RNA Later Weight-0.94	<0.0001	0.97
Pupa	Weight=0.9541*RNA Later Weight+0.23	<0.0001	0.98

Non-Maturing Larvae.

During the collection of larvae described in Generalized Additive Models, as well as in the blind study, a small percentage of individuals remained as postfeeding third instar larvae, even after adults were eclosing from their cohort. Such individuals could be problematic in a death investigation, as the collection of developmentally arrested (or "Peter Pan") individuals would mislead investigators into under-aging them if no pupae were observed/collected at the scene, resulting in an artificially short PMI estimate. The ability to detect "Peter Pan" flies would be important, eliminating those individuals from a prediction and might indicate that pupal samples were missed during evidence collection. "Peter Pans" were collected from each cohort (in Generalized Additive Models), as well as those observed in the blind study, and their gene expression profiles were compared to normal flies.

Methods

Ten (or as many as could be collected) "Peter Pan" larvae were collected from each replicate (see Generalized Additive Models above), which were fixed in 250 µL RNAlater (Applied Biosystems) and stored at –80ºC. Gene CT plots of non-pupating larvae from 55 individuals were organized by stage, to determine if any genes exhibited notable expression differences between normal postfeeding third instars and "Peter Pans", most notably if the middle 50% of CT scores for a gene in postfeeding third instars was beyond the median CT score for that gene in "Peter Pan" larvae. All plots were made in R (R core development team). F-tests (using the type III ANOVA command in R) were used to compare gene expression levels between feeding and postfeeding third instars, as well as between postfeeding third instars and "Peter Pan" larvae.

Results

Four genes (*hsp90*, *rop-1*, *sll*, *usp*) were identified as having expression patterns that might be used to differentiate "Peter Pan" larvae from normal postfeeding third instars. Expression plots (by stage) for these genes were compared to their counterpart plots in the blind study (Figures 1–4). Of the four genes that differed among the 55 known non-pupators, three of them (*sll*, *usp*, *hsp90*) expressed similar, abnormal patterns in blind study "Peter Pans", and could have been used to identify them as individuals that should not be incorporated into a PMI estimate.

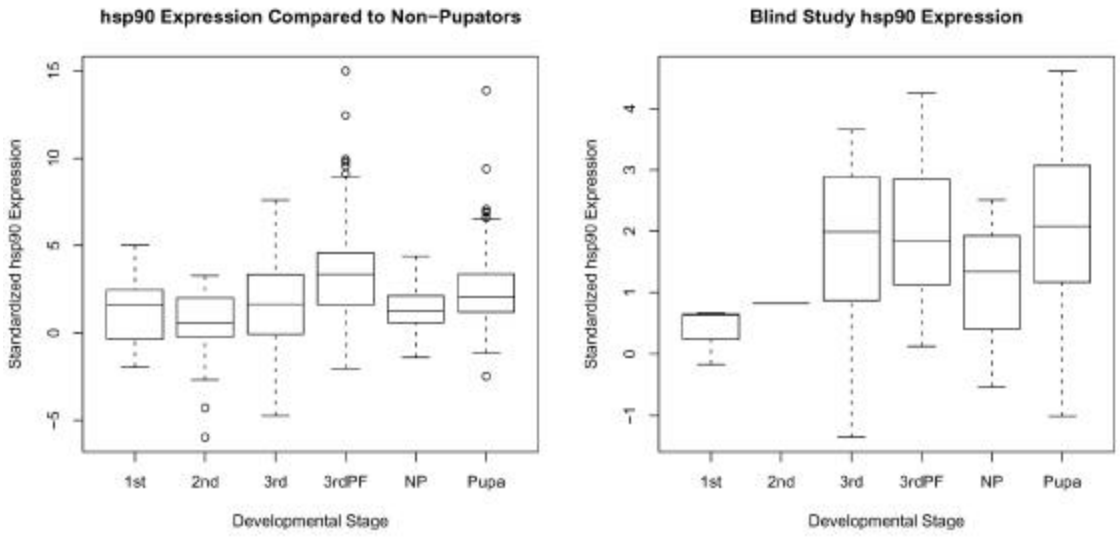

Figure 1. Standardized gene CT scores for *hsp90*, plotted by stage, for all individuals from the previous section, for the 55 non-pupator individuals from this study, and the individuals in the blind study. The left panel represents expression from the previous section, with results from the 55 non-pupators placed next to postfeeding third instars. The right panel represents gene expression for the individuals in the blind study, including the 4 non-pupators. 1^{st} = first instar. 2^{nd} = second instar. 3^{rd} = feeding third instar. 3rdPF = postfeeding third instar. NP = non-pupator. On average, *hsp90* was expressed at higher levels in non-pupators than in postfeeding third instars.

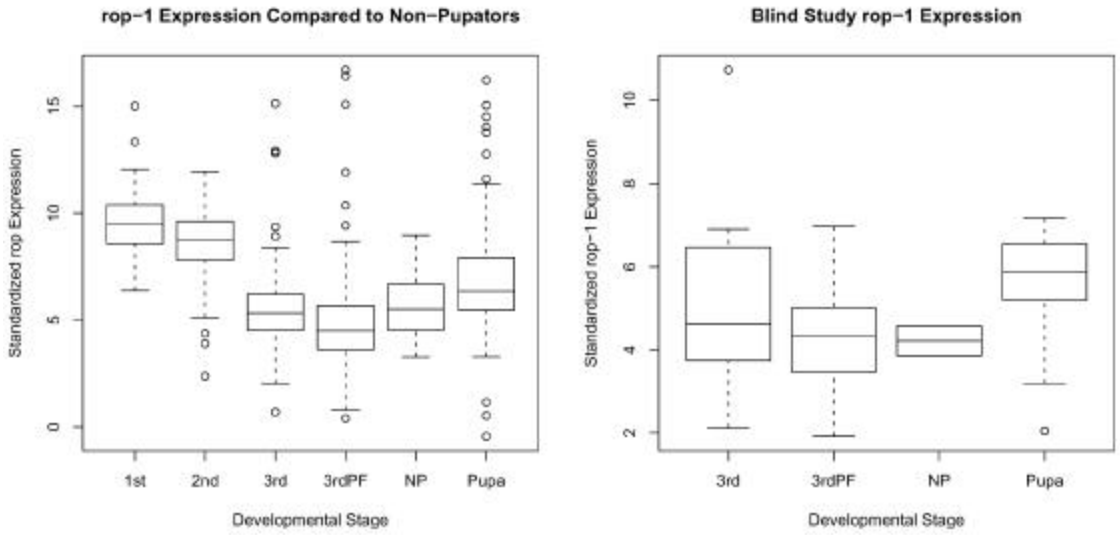

Figure 2. Standardized gene CT scores for *rop-1*, plotted by stage for all individuals from the previous section, for the 55 non-pupator individuals from this study, and the individuals in the blind study. The left panel represents expression from the previous section, with results from the 55 non-pupators placed next to postfeeding third instars. The right panel represents gene expression for the individuals in the blind study, including the 4 non-pupators. 1^{st} = first instar. 2^{nd} = second instar. 3^{rd} = feeding third instar. 3rdPF = postfeeding third instar. NP = non-pupator. On average, *rop-1* was expressed at lower levels in non-pupators than in postfeeding third instars, though this pattern was not apparent in the blind study.

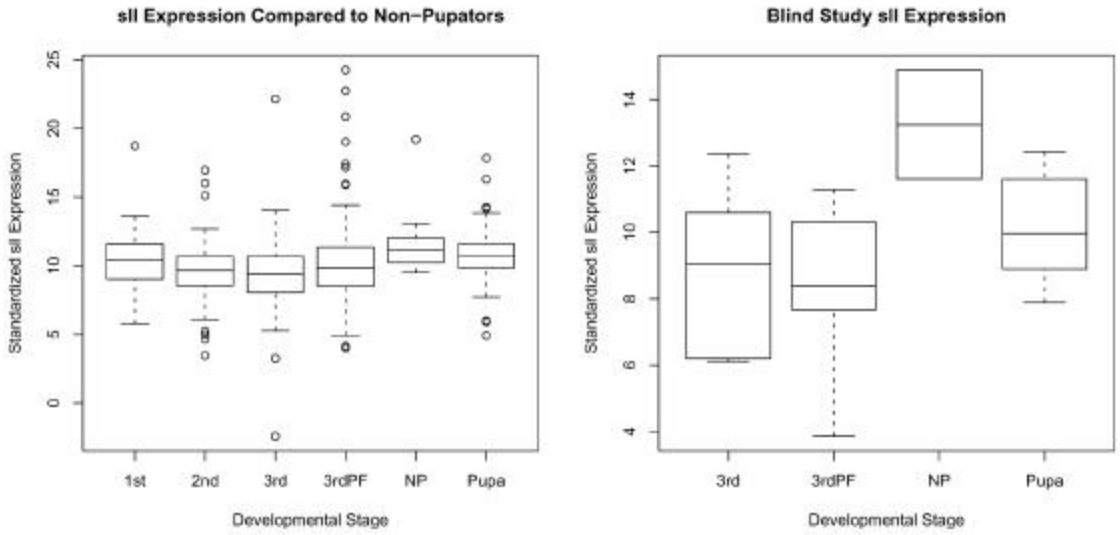

Figure 3. Standardized gene CT scores for *sll*, plotted by stage, for all individuals from the previous section, for the 55 non-pupator individuals from this study, and the individuals in the blind study. The left panel represents expression from the previous section, with results from the 55 non-pupators placed next to postfeeding third instars. The right panel represents gene expression for the individuals in the blind study, including the 4 non-pupators. 1^{st} = first instar. 2^{nd} = second instar. 3^{rd} = feeding third instar. 3rdPF = postfeeding third instar. NP = non-pupator. On average, *sll* was expressed at lower levels in non-pupators than in postfeeding third instars.

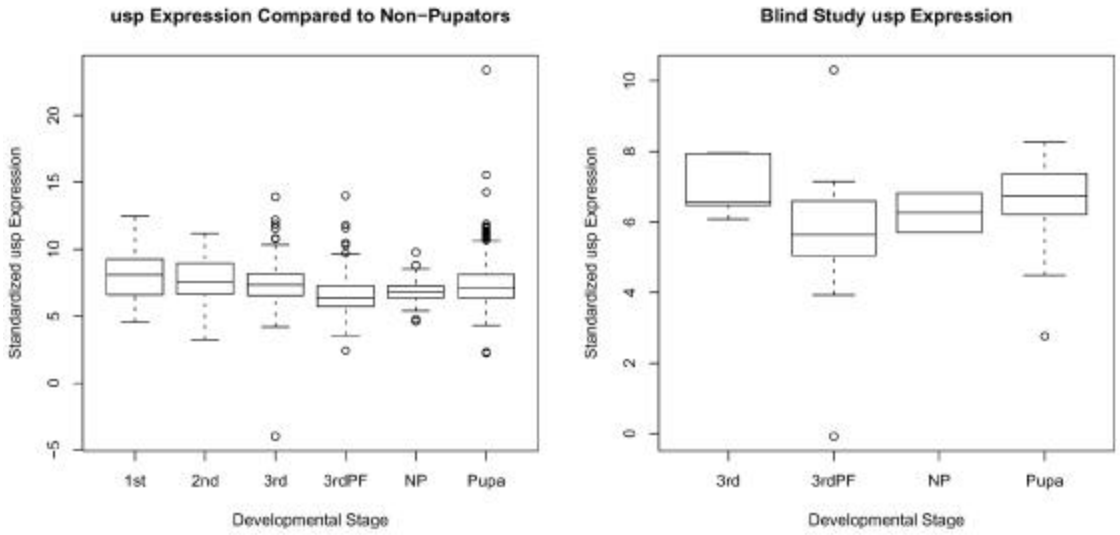

Figure 4. Standardized gene CT scores for *usp*, plotted by stage, for all individuals from the previous section, for the 55 non-pupator individuals from this study, and the individuals in the blind study. The left panel represents expression from the previous section, with results from the 55 non-pupators placed next to postfeeding third instars. The right panel represents gene expression for the individuals in the blind study, including the 4 non-pupators. 1^{st} = first instar. 2^{nd} = second instar. 3^{rd} = feeding third instar. 3rdPF = postfeeding third instar. NP = non-pupator. On average, *usp* was expressed at lower levels in non-pupators than in postfeeding third instars.

Statistical differences in gene expression among feeding, postfeeding, and non-pupating third instars were evaluated (Table 1). Expression profiles in feeding and postfeeding larvae revealed a group of genes that were differentially expressed when compared to postfeeding and "Peter Pan" larvae. Three of the four genes that were graphical different in expression in non-pupating postfeeding third instars (*hsp90, sll,* and *rop-1*) were shown to vary significantly. Another gene (*hsp60*) also differed significantly in "Peter Pan" larvae, but the change was subtle, making it an unlikely candidate for differentiating between larval types.

Discussion

The analysis of "Peter Pan" larvae indicates that two loci (*sll* and *hsp90*) stand out in their ability to eliminate such individuals from PMI estimates, thus avoiding the increase in error associated with their inclusion. These, along with *usp* exhibited reliable differences in expression patterns in the blind comparison. However, the difference in *usp* expression was not statistically significant with this sample size and will need to be confirmed. A fourth locus, *rop-1*, showed a significant difference in expression in "Peter Pan" larvae, however this was not validated in the blind study. Based on the results, low *sll* and *usp* abundance, along with high *hsp90* concentrations helped identify a "Peter Pan". Recognition of such patterns will also enable investigators to identify collections that missed pupae. For these reasons, the analysis of expression levels for *sll*, *usp*, and *hsp90* may be instrumental in determining the developmental status of a postfeeding third instar larva and whether or not it should be used to predict a PMI, though obviously further research will be required.

Table 1. Gene expression differences among third instar larvae. NS = not significant.

Feeding to Postfeeding		Postfeeding to Non-pupating	
Gene	*P-value*	*Gene*	*P-value*
cs	<0.0001	*cs*	NS
hsp60	<0.0001	*hsp60*	0.041
hsp90	<0.0001	*hsp90*	<0.0001
ace	NS	*ace*	NS
ecr	<0.0001	*ecr*	NS
rop	0.0013	*rop*	0.0151
w	0.0384	*w*	NS
usp	<0.0001	*usp*	NS (0.1752)
sll	NS	*sll*	0.0116

Overall Conclusions

The goal of the research detailed in this report was to improve the precision of blow fly age estimates, by including gene expression information in the process of predicting age. This goal was accomplished. Two basic areas of biological research were applied to the study of *L. sericata* development to achieve this goal. Quantitative genetic theory enabled a better understanding of the environmental and genetic effects on minimum development time and body size (detailed in Developmental Plasticity, Generalized Additive Models). More importantly, molecular biological approaches were used to examine gene expression changes and thus more precisely predict blow fly development percents. The predictable, age-dependent, variation of gene expression levels throughout the development of the species *L. sericata* was demonstrated. The variation in gene expression levels was then used to predict development percents using, now validated, GAMs.

These endeavors have helped to address current shortcomings of forensic entomological methods and PMI estimates. Introduction of standard operating procedures, particularly in the rearing of flies, the results from which PMI estimates are made, will not only make for a sounder product, but will help in meeting of Daubert requirements. Further, the use of GAMs, their related statistics, and their validation will enable investigators to describe and demonstrate the level of error in the models used to predict blow fly age. The ability to understand confidence intervals and error rates is another major tenet of Daubert, and research on this topic is underrepresented in the forensic entomology literature. Without these types of accomplishments, forensic entomological evidence could easily find itself removed from the courtroom, even though the science is well-accepted in its field.

Additionally, the use of gene expression data to predict a PMI provides technical qualities that make the approach more attractive for use in the death investigations than current methods. All expression analysis methods described herein can be applied in a laboratory prepared to do forensic DNA analysis. The use of robotics would enable the analysis of many samples in a rapid time frame. Likewise, the miniaturization of gene expression analysis can be accomplished through microarrays, which can be used to assess the expression levels of thousands of genes simultaneously. Most importantly, the gene expression data increased the accuracy of aging flies, particularly during the most troublesome portions of blow fly development, the post-feeding third instar, and pupa. It is these latter portions of development where the method will have the greatest impact. Taken together, the use of gene expression analysis to predict blow fly age is an attractive and viable approach for predicting a PMI with insect evidence.

Finally, the research presented opened up new avenues of exploration. Several aspects of the research demonstrated that gene expression analyses are useful for identifying otherwise unknown physiological/environmental conditions of the flies, many of which could lead to inaccurate estimates of PMI if not taken into account. For instance, the expression levels of two genes (*ace* and *hsp90*) were greatly altered depending on what temperature a replicate was exposed to. Knowing this, a scientist might gain information that could dramatically alter PMI estimates; that is, the temperature to which larvae were exposed. This information may not be otherwise readily available, such as in a closed environment. A skewed value for the two loci could immediately raise a warning flag. Similarly, the "Peter Pan" larvae, which failed to pupate in a timely fashion, could also throw off a PMI estimate, particularly if an officer was not trained to thoroughly look for pupae. Genetically, these individuals were readily identified through large differences in the expression levels of up to four genes (*hsp90, rop-1*, *usp,* and *sll*). Indeed,

numerous environmental conditions are known to affect gene expression, leaving the possibility of future research to identify loci that are molecular markers for a variety of environmental conditions affecting fly maturation. All of these will help to make forensic entomology a more thorough, accurate, and precise portion of the forensic sciences.

Tutorial on Predicting Blow Fly Age

R Statistical Program

The statistics analyzed in this research were done in the R statistical program (version 1.9.1; R core development team). This free statistics application has been developed by a dedicated group of statisticians and offers many of the features found in SAS. This section is designed to introduce readers to a very basic approach to predicting blow fly age with this program. Detailed information on the program can be found online, in Maindonald and Braun (2003) and Wood (2006), and with the **?** command followed by the application of interest (e.g. **?gam**).

R is based on the programming language S+. This is an object based program. An object is identified by its name then the equals sign, or an arrow (->), is used to define that object. For instance a linear model might be identified as follows:

Model1=lm(y~x1+x2+x3)

Model1 is the object defined by the equation y~x1+x2+x3, which is a linear model (identified by lm).

Data must also be entered in a specific manner in R. The method used herein was to identify the database as an object. That object was defined as a tab delimited text file as follows:

ls=read.delim("C:/Documents and Settings/Administrator/Desktop/Folder/R/Gam Work/All cDNA.txt",header=TRUE)

ls is the object.

read.delim identifies the file as a text file that is tab delimited.

header=TRUE indicates that column headings are to be kept.

In addition to loading a file into R and creating objects, it is also necessary to access libraries within the program to use specific statistical applications. Generalized additive models

are accessed by the command "library(mgcv)". Once the sample data are loaded into R and mgcv is loaded, it should be possible to create and use generalized additive models.

Sample Code

```
ls=read.delim("C:/Pathway/All cDNA.txt",h=T)
attach(ls)
```

These commands load the file and identify it as the default file for analysis.

Typing "ls" will show the database. Be careful, the program is capital sensitive.

summary(ls) will show a summary of all terms in "ls"

```
Percent=Hours.Old/Total.Dev.Time

cs=CS-((Tubulin+Rp49)/2)
hsp60=Hsp60-((Tubulin+Rp49)/2)
hsp90=Hsp90-((Tubulin+Rp49)/2)
ace=AcE-((Tubulin+Rp49)/2)
ecr=EcR-((Tubulin+Rp49)/2)
rop=Rop1-((Tubulin+Rp49)/2)
w=W-((Tubulin+Rp49)/2)
usp=Usp-((Tubulin+Rp49)/2)
sll=Slalom-((Tubulin+Rp49)/2)

library(mgcv)
```

Percent is the object that defines minimum development percent.

Each gene CT score is standardized by the equation noted above.

library(mgcv) loads the library with a generalized additive model application.

A GAM to predict development percent can be created as follows:

```
Test1=gam(Percent~Stage+s(Length)+s(Weight)+s(cs)+s(hsp60)

+s(hsp90)+…,family=Gamma(link=log))
```

Test1 is the object name for the model predicting Percent. This value is predicted with developmental stage plus smoothing curves (denoted by the "s") for length, etc. The gamma family with a log link function was applied to the model. No family identification is necessary for the gaussian distribution, as it is the default family.

The GAM model "Test1" can then be assessed as follows:

summary(Test1) will provide GCV, PDE, and sample size information.

anova(Test1) will provide the significance for each term as well as df and edf.

plot(Test1) will provide smoothed curve plots.

gam.check(Test1) will provide diagnostic plots, which can help in assessing error for that model as well as indicate whether the correct distribution was used.

Example

The GAM can then be used to predict Percent as follows:

-New data can be introduced as an object.

newd=data.frame(Stage="Pupa",Length=7,Weight=29.23,chs=5.73,hsp90=3.06,ecr=0.56,Temp=20, binchs=1,binace=0,binw=0,binsl=0)

-newd is the object for a data frame with the following data, which are the scores for one individual. Note that numerical values do not require the quotations, but the stage designation does.

After a model has been made and new data has been presented, the GAM can be used to predict Percent with the following command:

pred=predict.gam(Test1,newd,"response",se=T)

-pred is a new object that predicts GAM values.

-Test1 indicates the model used to predict Percent.

-newd indicates the data used to predict the Percent value.

-"response" indicates that the predicted value (Percent) is to be returned.

-se=T returns standard error estimates.

Appendix

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The graduate research was featured at the Science Museum of Minnesota, May–July 2006.