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PAPER

PATHOLOGY/BIOLOGY

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Gene Expression During Blow Fly Development: Improving the Precision of Age Estimates in Forensic Entomology*,†

ABSTRACT: Forensic entomologists use size and developmental stage to estimate blow fly age, and from those, a postmortem interval. Since such estimates are generally accurate but often lack precision, particularly in the older developmental stages, alternative aging methods would be advantageous. Presented here is a means of incorporating developmentally regulated gene expression levels into traditional stage and size data, with a goal of more precisely estimating developmental age of immature *Lucilia sericata*. Generalized additive models of development showed improved statistical support compared to models that did not include gene expression data, resulting in an increase in estimate precision, especially for postfeeding third instars and pupae. The models were then used to make blind estimates of development for 86 immature *L. sericata* raised on rat carcasses. Overall, inclusion of gene expression data resulted in increased precision in aging blow flies.

KEYWORDS: forensic science, *Lucilia sericata,* Calliphoridae, RNA, generalized additive model, forensic entomology, gene expression, reverse transcription PCR

Entomological evidence has long been used to aid investigators in the estimation of a postmortem interval (PMI; [1,2]). Such estimates are possible because necrophagous flies, especially blow flies (Diptera: Calliphoridae), are capable of colonizing remains soon after death and progress through a well-understood set of developmental stages, including the egg, three larval instars, pupation, and eclosion as an adult fly. Although the fundamental method for estimating blow fly age has remained unchanged for decades (reviewed in [2]), there are widely recognized limitations associated with it. The duration of each stage lengthens as blow fly development progresses, meaning increasingly larger windows of time must be placed around an age estimate, particularly for the postfeeding third instar and pupae, resulting in far less precise estimates of fly age. For example, the first instar of the blow fly *Lucilia sericata* can last as little as 24 h at 20°C, while at the same temperature pupation takes a minimum of 209 h (3). Temperature exerts the greatest influence during later developmental stages; the duration of the postfeeding third instar ranges from minimums of 82 h at 34°C to 200 h at 17°C, while pupation has minimums of 120–442 h at those temperatures, respectively (3).

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Consideration of immature fly length and weight (i.e., body size) can improve the precision of age estimates, but only for first, second, and early third instars, when the larvae are actively growing. Once third instars cease feeding they begin to shrink and exhibit increased variance in body size (3–5), adding to the difficulty of using size to estimate age (6–8). Likewise, size measurements do not enhance pupal age estimates as they do not change during development. Finally, difficulties in distinguishing between feeding and postfeeding third instar larvae (discussed in [5]) can compound the problem because age predictions of undetermined third instars must include estimates for the entire stage.

New approaches to predicting age of later blow fly developmental stages seem warranted, given the inherent difficulty in generating a precise PMI from this period. Toward this end, gene expression levels represent a novel data source during fly maturation. As an organism develops myriad proteins are temporally required (9), with the genes encoding them being up- or down-regulated. Among insects, gene regulation during development has been most extensively studied in *Drosophila melanogaster* (Diptera: Drosophilidae, e.g., [10,11]). Similarities in the molecular, genetic, and physiological makeup of the Drosophilidae and the Calliphoridae (12–16) mean that the *Drosophila* model can be used to target blow fly genes with an *a priori* expectation of informative regulation. Further, the general principle of aging blow flies via gene expression has already been demonstrated in one of the shorter developmental stages, the egg (17).

The research detailed herein was designed to address the hypothesis that estimates of blow fly developmental age could be made more precise by including gene expression data in the prediction process. The blow fly *L. sericata* was chosen because it is globally distributed and forensically informative (3–5,18) and has been studied at the molecular level in several instances (e.g., [16]), as has its sister species *L. cuprina* because of the role of the latter in myiasis

on sheep (19). This meant *L. sericata* gene sequences (for polymerase chain reaction [PCR] primers) could be obtained from the literature or generated based on *L. cuprina* data. Expression profiles of 11 genes were produced from a time-series collection of immature *L. sericata* comprising 958 individual larvae and pupae. The resulting data enabled the evaluation of gene expression as a means of predicting blow fly age using generalized additive models (GAMs; [8,17,20,21]). Finally, the utility of the models was assessed in a blind study, testing their ability to predict blow fly development.

Materials and Methods

Fly Rearing, Sequencing, and Gene Expression

L. sericata rearing methods and species confirmations were detailed previously (8,17,22). Multiple strains and environments were studied to evaluate the need to account for quantitative genetic influences on blow fly age estimates (23–25). Females from strains established in the spring/summer of 2005 from Davis, CA, East Lansing, MI, and Morgantown, WV, were allowed to oviposit for 1 h, after which egg masses were collected and split between rearing chambers at 20 and 33.5°C. Larvae were provided with beef liver on a moist paper towel until they achieved the postfeeding third instar, whereupon individuals with no visible tissue in their crops were transferred to clean sand at a density of 125 larvae per 500 mL sand. Ten larvae were collected in the morning and evening, body size measurements were taken (8,22), and flies were frozen at −80°C in RNAlater (Applied Biosystems, Foster City, CA; [17]) within a half hour. Five to 10 pupae (depending on pupal availability) were collected each morning. Age in hours, length, weight, and developmental stage were recorded for 958 flies, representing a subset of those studied in Tarone and Foran (8). The percent of development completed (age in hours/minimum total development hours; [8]) was also calculated.

Five flies from the 958 detailed earlier were evaluated for each sampling period from the Michigan and California strains raised at 20 and 33.5°C and from the West Virginia strain raised at 33.5°C. RNA was isolated in a 96-well format on an ABI PRISM 6100 (Applied Biosystems). Flies were homogenized by hand in 300 μL of RNA lysis solution (Applied Biosystems) using a sterile pestle. Lysates from pupae and larvae greater than 10 mg were diluted by adding 20 μL of larval lysates or 40 μL of pupal lysates to 300 μL of additional RNA lysis solution. The lysates were drawn through a 96-well filter plate (Applied Biosystems) to remove larval cuticle and pieces of puparium before being added to the RNA isolation plate. All other steps followed the manufacturer's protocol, using a final RNA elution volume of 100 μL. RNAs were DNase I treated as described (17).

Published *L. sericata* sequences were available for *ribosomal protein 49* (*rp49*), *slalom* (*sll*), *wingless* (*wg*), *heat shock protein* 60 and 90 (*hsp60*, *hsp90*), and *resistance to organophosphate 1* (*rop-1*) (http://www.ncbi.nlm.nih.gov/sites/entrez [accessed November 11, 2010]). Published *L. cuprina* or *D. melanogaster* sequences (http://www.ncbi.nlm.nih.gov/sites/entrez [November 11, 2010]) were used to design PCR/sequencing primers for *ß Tubulin 56 D* (*ßtub56D*), *chitin synthase* (*cs*) (both detailed in [17]), *acetylcholine esterase* (*ace*), *ecdysone receptor* (*ecr*), *ultraspiracle* (*usp*), *white* (*w*), and *scalloped wings* (*scl*) (Table 1), targeting 300 bp or larger gene segments. Sequencing methods were described previously (17), with minor adjustments made to annealing temperatures, extension times, and the number of amplification cycles (not shown). BLAST (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi [November 11, 2010]) comparisons confirmed that each gene was correctly assayed. Real-time PCR (RT-PCR) primer sequences were then designed as described (17).

cDNAs and reverse transcriptase-free controls were produced as detailed in Tarone et al. (17), using a High Capacity cDNA Archive kit (Applied Biosystems) according to the manufacturer's instructions. Reverse transcriptase negative controls were assayed with *rp49* primers (Table 1) to confirm the absence of genomic DNA. Positive controls utilized *rp49* primers and a cDNA sample derived from RNA that was pooled from each developmental stage. These were run on every plate, as was a negative control for the primer pair of the gene assayed on each plate. RT-PCR was performed as in Tarone et al. ([17]; see Table 1 for primer concentrations), by transferring reactions to a 384-well tray using a Biomek 2000 Automated Workstation (Beckman Coulter, Fullerton, CA). Ten microliter reactions containing 2 μL of cDNA and 5 μL Power SYBR Green PCR Master Mix (Applied Biosystems) were run on an ABI 7900 (Applied Biosystems). Only reactions yielding a dissociation curve exhibiting a single peak of the appropriate size were evaluated. Reactions were conducted in duplicate and cycle threshold (CT) values averaged. The CT for a gene was standardized against the two housekeeping genes, *rp49* and *ß tub 56D*, by subtracting the average of the housekeeping CTs from the average gene CT. In addition, reactions were standardized against each other by equalizing the CT for the positive control reactions.

Blind Predictions of Developmental Age

New MI *L. sericata* were collected in May of 2006 for use in a blind study predicting developmental age, with a goal of avoiding the effects of inbreeding resulting from over-winter rearing. Egg masses from these flies were deposited on the mouths of three CO_2 asphyxiated rat carcasses as described in Tarone and Foran (22); all rat usage followed Michigan State University's Institutional Animal Care & Use Committee guidelines. Carcasses were placed in incubators at 20, 33.5°C, or in a terrarium under outdoor ambient conditions in Okemos, Michigan from August 9–23 (average temperature 23.1 ± 8.9°C). The terrarium was covered with screening to allow air circulation and exclude other insects. One or two larvae/pupae were collected daily and stored in RNAlater (described above) by an independent investigator until first eclosion, which was considered minimum (100%) development time; 86 individuals were analyzed overall. Outdoor temperature was recorded using a HOBO data logger (Onset Computer, Bourne, MA), and accumulated degree hours (ADH; base temperature 10°C) were calculated (1) and used to determine development percent (age in ADH/minimum total ADH). Subsequent estimates of fly ages, using traditional and the molecular methods detailed above, were conducted blind. Predictions of stage were made visually, with spiracular differences used to identify the larval instars. The presence/absence of visible food in the crop was used to distinguish feeding from postfeeding third instars. In the five instances where larvae were recorded as feeding third instars during collection, but determined to be postfeeding based on crop appearance, the feeding stage identification was used, as investigators would be unable to know if they had misidentified a stage. A regression analysis of percent ADH by percent age in hours was then performed using Type III analysis of variance (ANOVA) to determine how well the two measures of development compared.

Body size measurements used to develop GAMs were based on live *L. sericata*, while the individuals in the blind study were stored in RNAlater before size measurements; therefore, it was necessary

TABLE 1—*Quantitative PCR and sequencing primers used in these experiments.*

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	EU089779	66.67
qAcE R	Quantitative PCR	TGATCCCAAAGGCCAACATT	EU089779	400
EcR F4	Sequencing	TTTCACCCTCGAGCAGTCTT	U75355	1000
EcR R3	Sequencing	CTTTCTTTTCGCGTCGTTTC	U75355	1000
qEcR F	Quantitative PCR	GCATGCGGCCGGAAT	EU089777	400
qEcR R	Quantitative PCR	GCGTCGTTTCATTGCACACT	EU089777	400
Usp F1	Sequencing	CGCAGGAGATAAAGCCAGAC	AY007213	1000
Usp R1	Sequencing	TGGTGTCGACGTGCATATT	AY007213	1000
qUsp F	Quantitative PCR	CGAGCAAAAAGCCGAATCAC	EU089778	400
qUsp R	Quantitative PCR	TGCCTACGCGCAAAAAGG	EU089778	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	EU089776	400
qW R	Quantitative PCR	GCGCCCAGTGTCCTACCA	EU089776	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	EU089780	400
qScl R	Quantitative PCR	TTCTCCGGGATTGGTGACA	EU089780	400

to convert stored size to live size. Individual larvae (n = 400) from the MI strain that had been measured live were remeasured and reweighed after approximately 1 year of storage. Linear regressions (26) were used to convert the sizes of stored flies to estimated live sizes.

Statistical Analyses of Blow Fly Development

Statistics and graphs evaluating fly development were produced using the R statistical package (26). Type III nested-ANOVAs were used to determine whether gene expression changed significantly during development, nesting fly strain within rearing temperature, which was nested within temporal blocks that consisted of cohorts laid on the same day. The *ace* locus displayed unique expression patterns that could not be explained through these standard models (see Results), thus other interactions were examined for it alone. For all loci, both developmental stage and development percent were considered. A Bonferroni correction was applied as multiple variables were tested in each model; accordingly, a p-value of 0.0125 was considered significant. Standardized CT values for each gene were plotted against minimum development percent, and locally weighted sum of squares curves were drawn through the nonlinear data, allowing comparisons of average gene expression between temperature treatments or among strains.

GAMs were developed to assess the influence of body size, developmental stage, rearing temperature, fly strain, and finally gene expression, on predicting age. Two measures of gene expression were evaluated: quantitative differences in CT values for all genes, or the ability to simply detect expression of a gene (binary data where 0 = off and 1 = on). Four GAMs were examined: Model 1 (the control model) included standard entomological data, encompassing body length, weight, developmental stage, rearing temperature, and fly strain, to which other models were compared; Model 2 included all terms in the first model plus gene expression data; Model 3 anchored gene expression levels with length data (21); and Model 4 removed strain and temperature from Model 3 to examine their influence (expression anchored to length only is presented because anchoring it to weight did not decrease error in GAM predictions). Generalized cross-validation (GCV) and percent deviance explained (PDE) statistics were also generated, with superior models yielding lower GCV and higher PDE scores. Two diagnostic plots were used to evaluate and compare error for each model. The first was histograms of residuals, which estimate error by subtracting an actual age from the predicted age. Models exhibiting no more than 10% error were considered highly accurate. The second set of plots graphed actual age against predicted age, which was useful for identifying periods of development with high error (such as the later developmental stages discussed above) or periods of development that a model is not capable of accurately predicting. Plots of true versus predicted ages of the blind study flies were considered accurate if their deviation from true development percent was within 10%. Gamma or Gaussian distributions were

applied depending on which better satisfied the assumptions of error (21).

GAMs based on the original (nonblind) data were also evaluated for the postfeeding third instars and pupal stages using both continuous and binary gene expression, as these stages provide the least precise age predictions with traditional data. Length-anchored models could not be considered for stage-specific GAMs because of smaller sample sizes. Length to weight ratio of the postfeeding third instar model was not tested for the same reason.

Development percent was first predicted for the 86 blind study flies based on standard measurements of body size and developmental stage (the control). These included 34 flies reared at 20°C, 29 reared at 33.5°C, and 23 reared at outdoor summer ambient temperature. Seven flies were identified as first instars, four as second instars, 15 as feeding third instars, 18 as postfeeding third instars, and 42 as pupae. The 71 individuals that yielded gene expression data allowed predictions of developmental age using GAMs similar to Models 2–4, except that strain was excluded as only one strain was examined. Temperature was included for flies raised at a constant temperature but not for the ambient temperature treatment because fluctuating temperature was not incorporated in model development. Blind prediction models were classified as Type A when they contained little or no gene expression data, resulting in diagnostic plots that exhibited increasing error during development as occurs in traditional forensic entomological data (Model 1; [8]) and as Type B if more extensive gene expression data were available and plots showed a relatively even distribution of error during development. Age predictions incorporating gene expression data were then compared to predictions based solely on body size and developmental stage. Predictions were considered accurate if they were within 10% of true age.

Error distribution (the absolute range of under- and overestimation) was evaluated for three basic models in the blind study: the control model, models using all available gene expression data (Type A and B combined), and those containing only substantial gene expression data (Type B), to characterize the effects of each on the precision of age estimates. The error in Type B predictions was also evaluated for each temperature treatment. Error in age estimates (predicted minus true development percent) was compared using box plots, which display the median error as a bar, the middle quartiles as a box, and the 99th percentiles as whiskers.

Results

Species Identification and Development

Flies collected in 2005 were previously identified as *L. sericata* based on both visual examination and 655–776 base pairs of *cytochrome oxidase 1* sequence data (NCBI accession numbers DQ868503, DQ868523, and DQ868524 for CA, MI, and WV, respectively [8,22]). A 686-bp sequence from the 2006 MI strain (accession number EU848424), used in the blind study, was identical to published *L. sericata* sequence. Nuclear genes sequence comparisons (Table 2) also showed the highest identity with published *L. sericata* data (where available), or 95% or greater identity with *L. cuprina*.

Gene expression profiles were generated for 958 individuals. After *c.* 100 had been profiled, *wg* and *scl* data showed high variation in expression and a lack of temporal change in mean expression, indicating they were unlikely to provide useful information; they were dropped from further study, resulting in nine genes for analysis. Overall, 48 first instar larvae, 79 second instar larvae, 135 third instar larvae, 334 postfeeding third instar larvae, and 362 pupae were analyzed. Two hundred and sixty CA and 272 MI flies reared at 20°C were examined, as were 149, 121, and 156 individuals reared at 33.5°C from the CA, MI, and WV strains, respectively. Full expression profiles were obtained for 501 flies, with the remainder generating data for a subset of the genes.

Expression of Informative Genes

An exhaustive description of the gene expression data can be found in Tarone (27) and Foran (28); the results presented here focus on the most pertinent data. Gene expression throughout development was evaluated graphically and statistically; average gene expression levels across strains and temperatures are displayed in Fig. 1. Type III nested-ANOVA indicated that all loci exhibited significant ($p \leq 0.0125$) linear temporal changes in expression levels, either based on the stage of development (*cs*, *ace*, *hsp 60*, *hsp 90*, *ecr*, *rop-1*, *usp*, *w*, *sll*) or as a function of development percent (*ace*, *hsp 60*, *hsp 90*, *rop-1*, *usp*, *sll*). Third instar larvae had several

TABLE 2—*Gene sequencing results.*

Gene	Species	Size	Percent
β tubulin 56 D	*Glossina morsitans morsitans*	635	86
chitin synthase	*L. cuprina*	713	98
acetylcholine esterase	*L. cuprina*	369	99
ecdysone receptor	*L. cuprina*	350	98
	L. sericata	102	100
ultraspiracle	*L. cuprina*	683	95
	L. sericata	508	99
white	*L. cuprina*	861	95
scalloped wings	*L. cuprina*	884	96

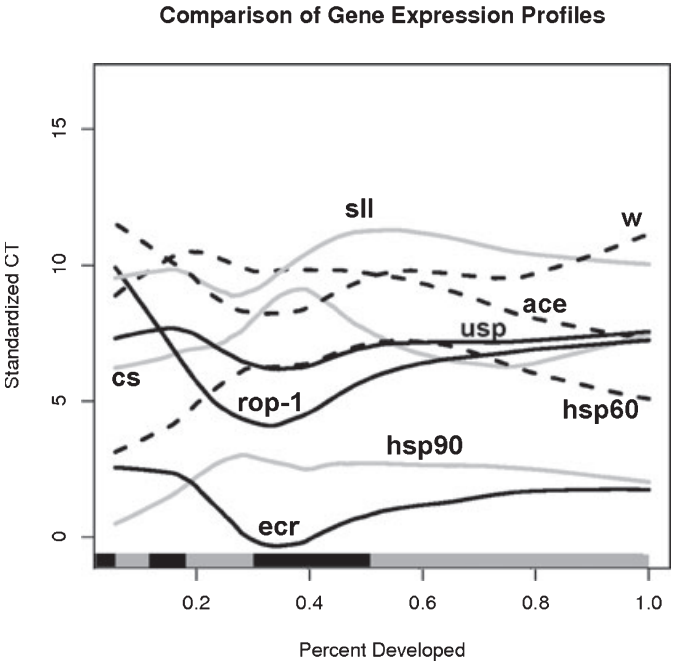

FIG. 1—*Standardized CT scores for the nine loci evaluated in 958 individuals sampled throughout development. Note that CT is inversely proportional to gene expression level. On the X-axis, 0 denotes when eggs were laid and 1 indicates eclosion of adults. The alternating gray and black bars along the X-axis indicate approximate durations of the egg, first instar, second instar, feeding third instar, postfeeding third instar, and pupal stages.*

genes that were significantly up- or down-regulated between feeding and postfeeding stages (*cs*, *ecr*, *hsp60*, *hsp90*, *rop-1*, *w*, and *usp)*. Four genes (*cs*, *ace*, *w*, and *sll*) were frequently not detected at some point in development ($p < 0.0125$), so their binary expression (on/off) data were also included in GAM analyses. *rop-1* was low or off at the very earliest developmental stage; however, it was detectable at all point thereafter, so was not included in the binary data.

Cohorts of flies laid on different days (temporal blocks) exhibited significant differences in gene expression levels, although the sizes of these effects were small, in that there was broad overlap in the distribution of CT values for each gene. In development percent models, all loci except *ecr*, *w*, and *sll* differed significantly in expression by block. When expression levels were considered by developmental stage, the same loci, plus *w* were significantly affected.

The expression of some genes was affected by rearing temperature and/or fly strain (Fig. 2). Significant temperature effects were detected in *cs*, *hsp 90*, *rop-1*, and *usp* when evaluating expression by developmental stage, and cs, *hsp 60*, *hsp 90*, *ecr*, *rop-1*, and *usp* based on development percent. Genes exhibited an array of responses to temperature, ranging from weak for *hsp60* (Fig. 2*a*) to strong for *hsp90* (Fig. 2*b*) and *ace* (not shown). In some instances, rearing temperature affected the developmental point at which gene expression reached its maximum or minimum: e.g., *rop-1*, for which high temperatures resulted in a faster attainment of maximum expression (data not shown). There were significant differences among strains in the expression of six genes (*ace*, *hsp 60*, *hsp 90*, *ecr*, *usp*, *sll*) in models encompassing developmental percent, and the same loci plus *cs* in models of developmental stage. Some of these differences were very small, with expression curves among strains overlapping at multiple points during development (e.g., *hsp60* at 33.5°C; Fig. 2*c*), while others had similar expression profiles over time, with only the relative amount of gene expression changing (e.g., *usp* at 20°C; Fig. 2*d*). Expression levels of *ace* were further evaluated to identify interactions between temperature, strain, and temporal block, as the former seemed to influence its expression although it was not significant when examining temperature alone. When expression of this locus was considered in terms of development percent, interactions between development percent, temperature, strain, and temporal block on *ace* trended toward significant ($p = 0.04$). Excluding strain resulted in an effect of temperature and block ($p = 0.01$) on *ace* transcript levels.

Statistical Models of Blow Fly Development

The effectiveness of the four models in accurately estimating overall fly development is shown in Table 3. The three gene expression-based GAMs (Models 2–4) had lower GCV scores than the control. Likewise, they exhibited similar (no more than 0.5% smaller) or better (up to 2.8% larger) PDE scores. Finally, removing rearing temperature and fly strain from gene-based predictions (Model 3 vs. Model 4) resulted in a negligible change in GCV (0.0003) or PDE (0.2%), indicating that they exert little influence on development percent predictions with gene expression data.

Diagnostic plots demonstrating the age estimate improvement using gene expression data are shown in Fig. 3. The control model residuals (Fig. 3*a*) showed a broad distribution, indicating large differences between observed and predicted age. The source of much of this error becomes apparent when examining the response versus predicted development plots, wherein an ideal plot produces a straight line (i.e., $X = Y$). The control model was relatively precise during early development (the first *c.* 25%), but then produced a block of data points at *c.* 30–40% of development (postfeeding third instars), an incorrect gap in the data, another block of data points between *c.* 60% and 80% of development (pupae), and another incorrect gap in the data beyond that. Inclusion of gene expression data (Fig. 3*b*) tightened the residuals considerably, while helping to eliminate gaps in the data, thus increasing precision overall. The residuals for the length-anchored predictions yielded *c.* 90% of predictions within 10% of true development percent (Fig. 3*c*), and an even stronger relationship between response versus predicted age.

Examining the problematic postfeeding third instar and pupal stages individually (Fig. 4) shows the extreme improvement in age predictions gained when gene expression data were included. The control larval model residuals exhibited an age estimate range that was approximately two to three times as large as the gene-based model (Fig. 4*a*,*b,* respectively), with PDE values increasing from 33.4 to 76.6% through inclusion of expression data. The range of residuals in pupae was approximately twice as large for the control (Fig. 4*c*) as the gene-based model (Fig. 4*d*), and PDE increased from 13.6 to 73.2%.

Blind Predictions of Development Percent

Seventy-one of the 86 flies analyzed in the blind study produced gene expression data, 22 of which could be used in Type A models and 49 in Type B models; the rest did not produce gene expression data. Twenty-one of the Type B samples were third instars (six reared at 20°C, eight reared at 33.5°C, seven reared at ambient temperature) and the rest were pupae (12 reared at 20°C, 10 reared at 33.5°C, six reared at ambient temperature). Development percent in terms of ADH closely tracked development for flies raised in ambient temperature conditions; regression analysis showed percent ADH was defined as 0.996 times the percent age in hours, with a Y intercept of 1.245 ($p < 0.0001$ and $p = 0.0001$ for the model and intercept, respectively; $R^2 > 0.99$), indicating that ADH was an appropriate measure of developmental progress for the blind study. The length of larvae and pupae stored at −80°C in RNAlater was a significant predictor of live length ($p < 0.0001$), with R^2 values ranging from 0.59 (postfeeding third instars) to 0.89 (feeding third instar).

Plots of predicted versus actual development in the blind study are displayed in Fig. 5. The control model (Fig. 5*a*), predicted 60.5% of the flies to be within 10% of their true developmental age, and again displayed a gap in age estimates between *c.* 40–60% and after *c.* 80% of development. Seventy-seven percent of larvae were predicted within 10% of true development, while only 43% of pupae were predicted that accurately. Twenty-seven underestimates and seven overestimates of age occurred. Incorporating the gene expression data into predictions for the same 86 samples improved predictions to 61.6% overall and 79.5% for larvae (data not shown); there was no increase in accuracy for pupae, but the gap between larval and pupal predictions was smaller. The development of 26 flies was underestimated while seven were overestimated. When the 49 Type B predictions were considered (Fig. 5*b*), there were three overestimates and 24 underestimates. Predictions using length-anchored gene expression for all 86 individuals increased accuracy to 65.1% overall, with an increase to 79.5% for larvae, 50% for pupae, including 24 underestimates and six overestimates (data not shown). The 49 type B predictions with length-anchored expression data (Fig. 5*c*) yielded two overestimates and 23 underestimates.

Comparison of error from blind study age predictions of larvae and pupae (Fig. 6*a*,*b,* respectively) showed that incorporating gene

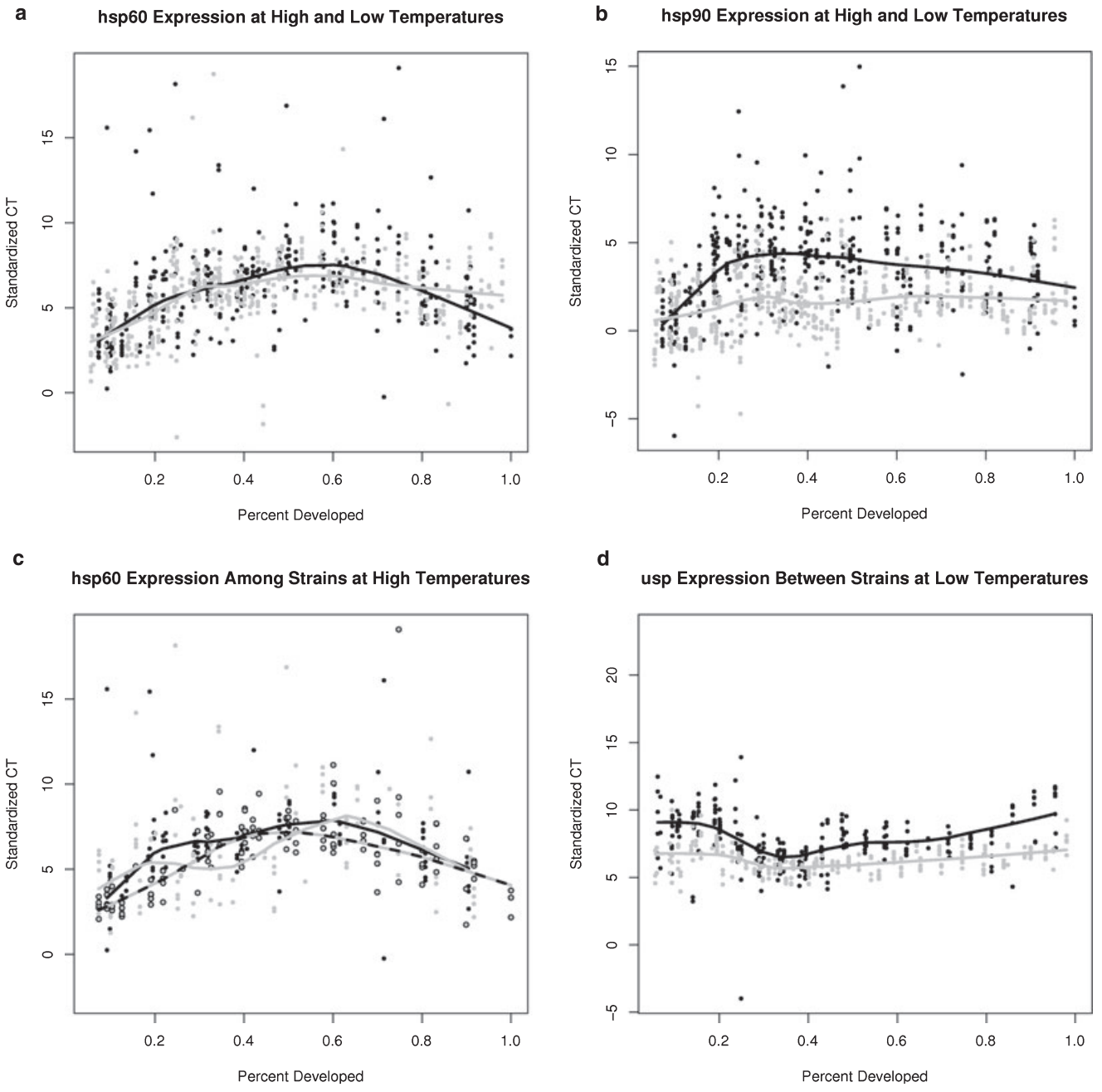

FIG. 2—*Variation in CT scores for different loci based on fly rearing temperature and strain. A range of responses is shown. (a) Some genes were expressed in similar patterns at both temperatures in all strains. Although statistically different between temperatures, hsp60 in all strains was expressed similarly at 20°C (gray) and 33.5°C (black). (b) Temperature resulted in large changes in the expression profile of some genes. hsp90 in all strains was expressed at notably higher levels at 20°C (gray) than 33.5°C (black). (c) Some genes were expressed at similar levels by all strains. hsp60 at 33.5°C, although again statistically different, was expressed similarly by the CA (gray), MI (black), and WV (gray and black) strains, with curves often crossing one-another. (d) Other genes showed similar expression patterns but at consistently varying levels in different strains. usp at 20°C was expressed at higher levels in the CA strain (gray) than in the MI strain (black).*

TABLE 3—*A comparison of the four models used to assess prediction of development percent with gene expression.*

Model	Model Parameters [distribution(link function)]	GCV	PDE	*N*
1	Stage + Strain + Temp + s(Length) + s(Weight) + s(Length,Weight) [gamma(log)]	0.038	91.8	958
2	Stage + Strain + Temp + s(L) + s(W) + s(L,W) + s(genes) + binary [gaussian(identity)]	0.0079	91.3	501
3	Stage + Strain + Temp + s(L) + s(W) + s(L,W) + s(Length,genes) + binary [gaussian(identity)]	0.0056	94.6	501
4	Stage + s(L) + s(W) + s(L,W) + s(Length,genes) + binary [gaussian(identity)]	0.0059	94.4	501

GCV, generalized cross-validation; PDE, percent deviance explained.

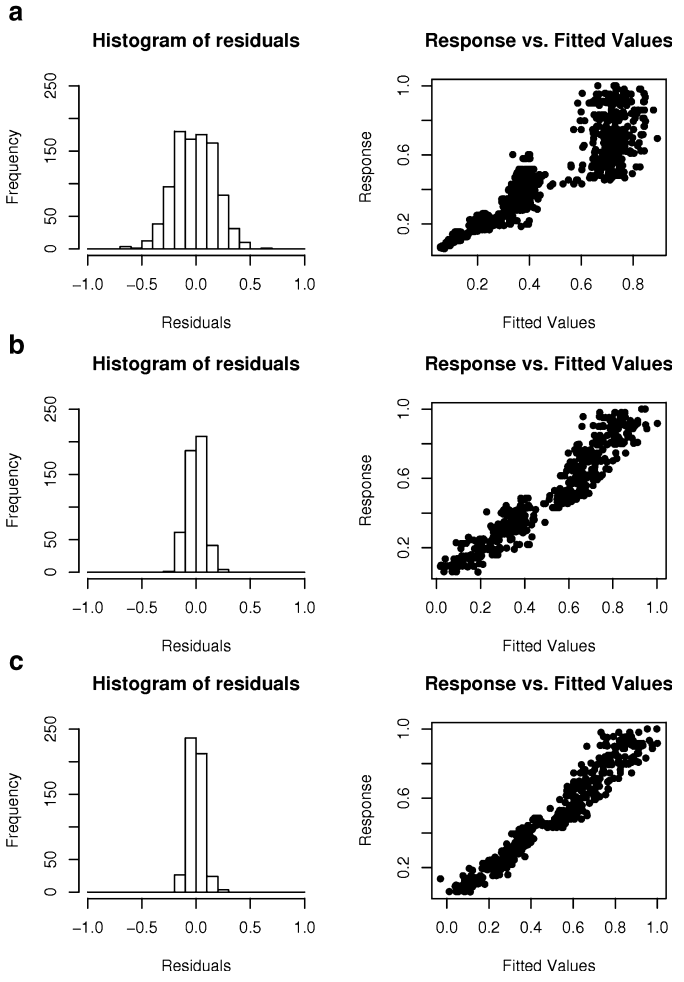

FIG. 3—*Representative error plots of GAMs used to predict development percent on a scale of 0–1. 0 indicates when eggs were laid and 1 indicates eclosion of adults. The left plot for each type of model is a histogram of residuals and the right plot depicts predicted (fitted) versus true (response) values for each model. (a) Error plots for Model 1, the control model, which used stage/length/weight/strain/temperature to predict development percent. Such models yielded increasing error over time, with gaps in predictions at* c. *40–60% and after 80% of development. These were designated Type A models. (b) Error plots for Model 2, which included the terms in Model 1 plus gene CT scores for the nine loci in Fig. 1 and on/off data for cs, ace, w, and sll. The range of residuals decreased and the gap in predictions between pupal and larval samples shrank with the inclusion of gene expression data. (c) Error plots for Model 3, which included the terms in Model 2, with gene CT scores anchored against length. Residuals decreased when compared to Model 2 and there was a relatively smooth transition from larval to pupal predictions. This pattern is also representative of the error plots for Model 4 (not shown), which did not include strain or temperature information in predictions. Models with error plots similar to Models 2–4 were designated as Type B.*

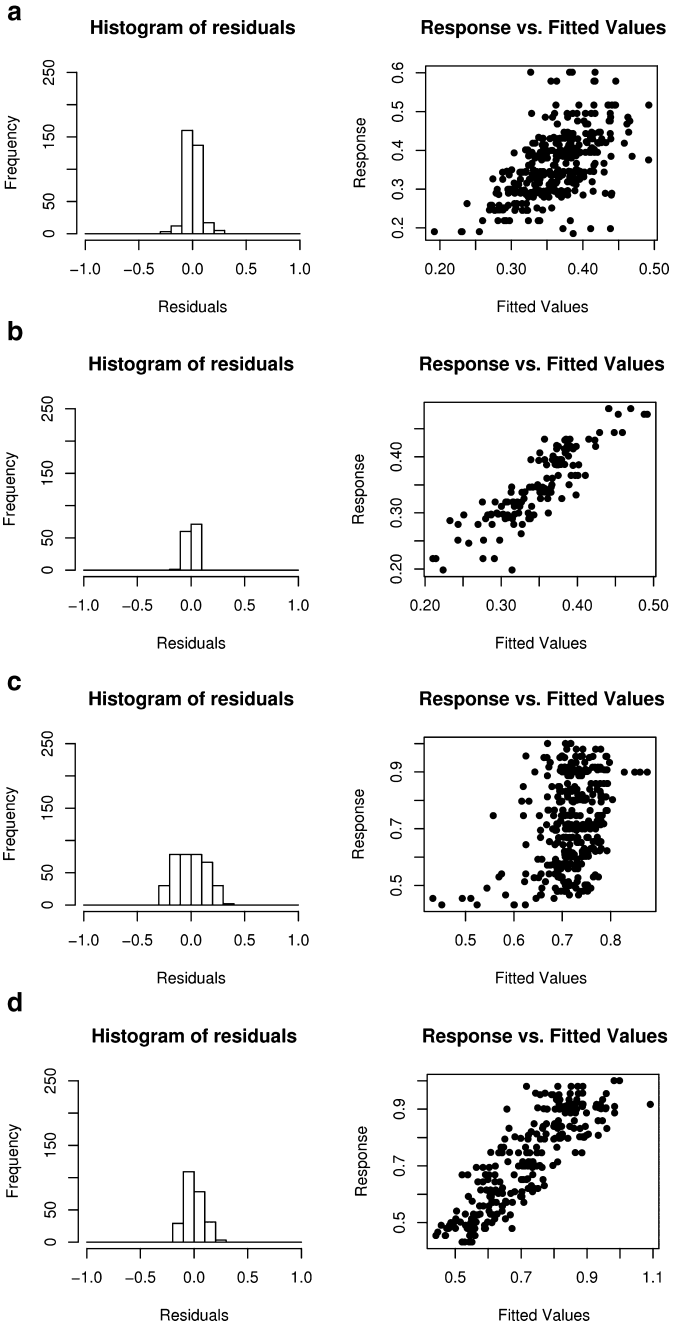

FIG. 4—*Error plots for GAM predictions of development percent in* L. sericata *postfeeding third instar and pupal samples. Histogram of residuals and fitted versus response plots are as in Fig. 3. (a) Error for a control GAM using body size in postfeeding third instars. (b) Error for gene expression GAM using the parameters in (a) and gene expression data. (c) Error for a control GAM using body size in pupae. (d) Error for gene expression GAM using the parameters in (c) and gene expression data. Note that the inclusion of gene expression decreased error for age estimates of both developmental stages.*

expression yielded a smaller range of error when full profiles were used to predict development percents, resulting in a 3–8% decrease in age overestimation. Larval controls (Fig. 6*a* Control), blind predictions including length-anchored gene expression data for all samples (Fig. 6*a* 1), and unanchored gene expression data (Fig. 6*a* 2) were all accurate, in that the true age was spanned by the predictions. Age estimates without length-anchored expression data (Fig. 6*a* 2) were more precise (had a smaller range of residuals) than length-anchored models. Predictions made with Type B models where gene expression was unanchored to length (Fig. 6*a* 3) resulted in the greatest increase in precision. When the Type B estimates were evaluated by temperature class (sample sizes were eight, six, and seven, for Fig. 6*a* 4–6, respectively), the 33.5 and 20°C predictions were more precise than the control and accurate in that they were within 10% of true age (Fig. 6*a* 4 and 5, respectively). However, while the ambient temperature group (Fig. 6*a* 6) yielded the smallest error range, only three of the ambient temperature predictions were within 10% of true age. The age predictions

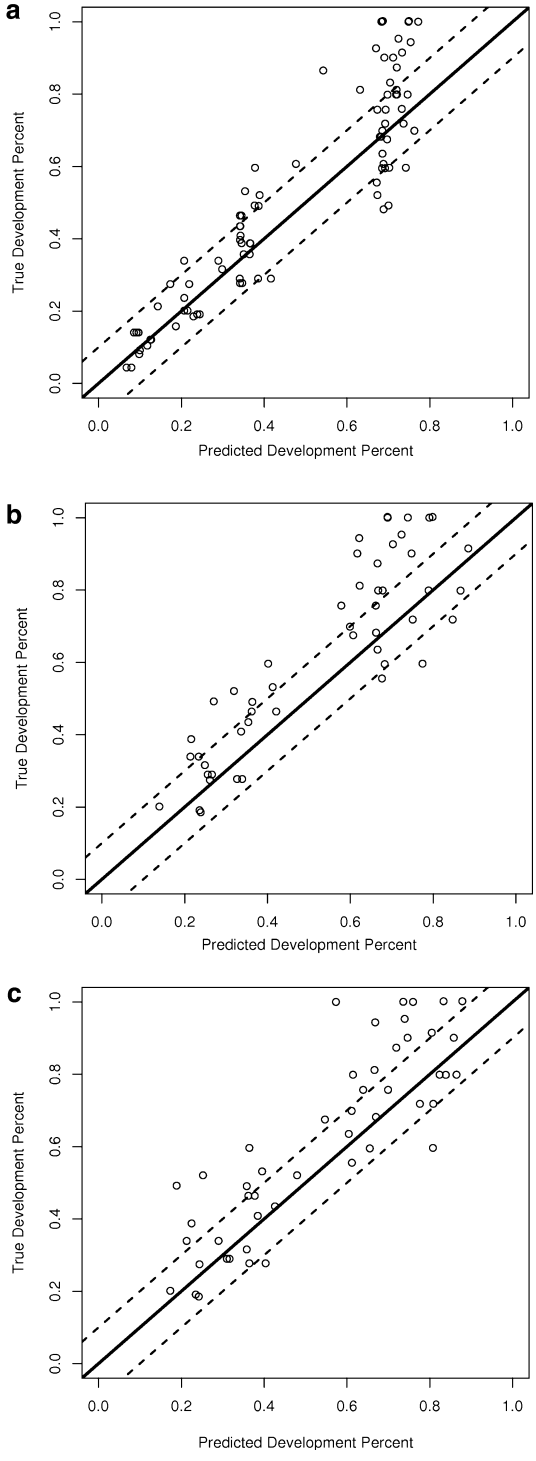

FIG. 5—*Blind predictions of* L. sericata *age with and without gene expression data where 0 indicates when eggs were laid and 1 indicates adult eclosion. Solid lines denote the predicted versus true development percent, dotted lines denote predictions within 10% of true. Strain was not considered in blind predictions. (a) Blind predictions of 86 individuals using control GAMs that included developmental stage, length, weight, along with temperature for the 20 and 33.5°C treatments. Predictions were made with GAMs that followed the format of Model 1. (b) Blind predictions of 49 individuals that yielded sufficient gene expression data to develop Type B models. Predictions were made with GAMs that followed the format of Model 2. (c) Blind predictions for the same individuals in b), using length-anchored GAMs that followed the format of Model 3. Estimates with gene expression data yielded more accurate pupal ages (approximately the last half of immature development) and predicted fewer individuals as >10% of their true development percent.*

for the five incorrectly identified postfeeding third instars were also evaluated to determine whether the misidentification of stage affected the accuracy of the predictions these samples produced. In all cases, they were within 10% of true development percent.

Predictions of pupal development (Fig. 6*b*) without (Fig. 6*b* Control) and with gene expression were accurate; however, inclusion of Type A models (Fig. 6*b* 1 and 2) caused a precision decrease. Type B models that did not use length-anchored gene expression data (Fig. 6*b* 3) improved precision. As with larval samples, the different temperature treatments (sample sizes were 10, 12, and six for Fig. 6*b* 4–6, respectively) yielded varying results. The 33.5 and 20°C predictions (Fig. 6*b* 4 and 5) generated precise estimates of blow fly age that spanned the true development percent, while the ambient temperature predictions (Fig. 6*b* 6) produced underestimates of development percent, with two estimates within 10% of true age.

Discussion

The experiments described herein were designed to examine whether gene expression data can be used to augment standard methods of aging developing blow flies, with a goal of more accurately and precisely approximating a PMI. Estimating fly age with relatively high precision is straightforward in early developmental stages (egg through second instar), given the short duration of each stage. In contrast, the far longer duration of the third instar and pupal stages makes aging them more difficult. Error stemming from these stages is exacerbated by the fact that size is not indicative of age during this period of development because of the highly variable reduction in length and weight of postfeeding larvae and no discernable change in pupal size. Given this, any developmentally related measurements that are all or in part independent of fly developmental stage or size have the potential to increase the precision of age estimates. In this regard, the myriad genes that are up- or down-regulated during fly development are potentially reliable markers of development.

Nine genes (*ace*, *cs*, *ecr*, *hsp60*, *hsp90*, *rop-1*, *sll*, *usp*, and *w*) evaluated in this study provided useful data, being differentially regulated during development. Seven of these (*cs*, *ecr*, *hsp60*, *hsp90*, *rop-1*, *usp*, and *w*) helped distinguish the morphologically similar, and thus problematic, feeding and postfeeding third instars. Research in *Drosophila* indicates that a pulse of the hormone ecdysone triggers the third instar switch from feeding to postfeeding, initiating metamorphosis through a cascade of gene expression changes (11,29,30). Consequently, it is not surprising that *ecr* and *usp*, which encode the heterodimeric protein that triggers ecdysone-mediated changes, shifted their relative expression levels during this period of development. Likewise, the other five genes exhibited expression changes that suggest they are also ecdysone responsive, with highest or lowest expression levels corresponding with peak *ecr* expression (Fig. 1).

Genotype and the environment are also known to influence gene expression (23), which was observed in the current data. The three fly strains expressed all loci at different relative levels, with the exception of *rop-1* and *w*. Further all loci except *w* and *sll* were expressed differently between temperature treatments. Most of these responses were extremely subtle however (e.g., *hsp60*, Fig. 2*a*), such that the variation in expression profiles was greater than the difference in mean expression among strains or between rearing temperatures, indicating that there was sufficient power to detect major developmental effects. More importantly, the results suggest that strain and temperature may not need to be considered when making predictions of fly development percents based on gene

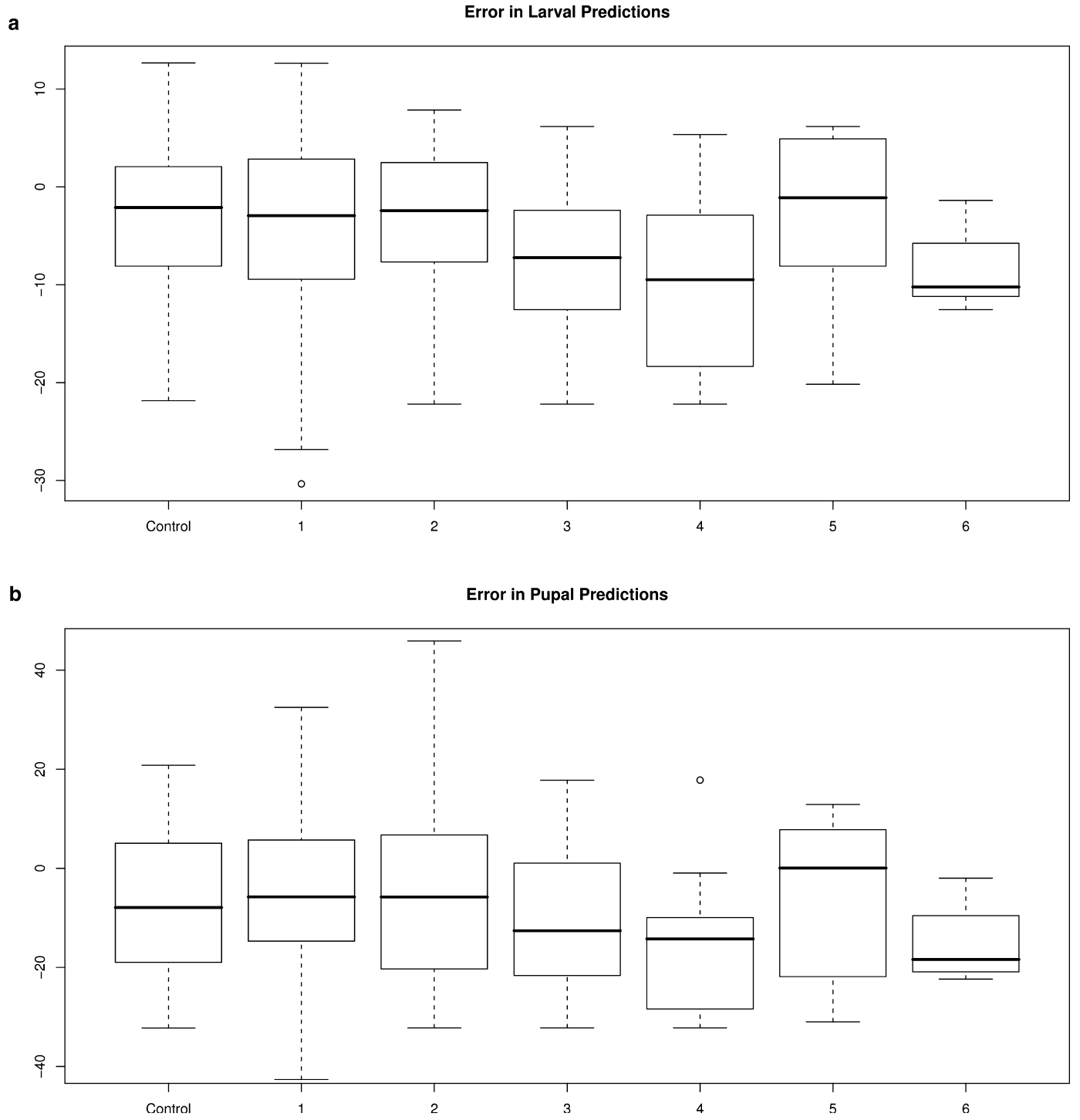

FIG. 6—*Box plots of error for blind predictions of development percent. Plotted values are the predicted development percent minus the true development percent, with positive values representing overestimates of age and negative values representing underestimates. Median error for blind predictions of development percent is represented by the horizontal bar, the middle two quartiles are represented by the box, and the 99th percentile are represented by the whiskers on the plots. (a) Error for larval predictions. (b) Error for pupal predictions. Control = predictions using length, weight, and developmental stage. 1 = predictions with Type A and B GAMs that included length-anchored gene expression information. 2 = predictions with Type A and B GAMs. 3 = predictions with Type B GAMs. 4 = Type B GAMs for flies that were raised at 33.5°C. 5 = Type B GAMs for flies that were raised at 20°C. 6 = Type B GAMs for flies that were raised at outdoor ambient conditions. Note the bias toward underestimates with all models.*

expression levels. The exceptions to this were *ace* and *hsp90*, which were both strongly down-regulated during the larval–pupal transition at high temperatures only, resulting in distinct temperature-dependent developmental profiles. As heat shock proteins change expression levels with temperature shifts (31), it is not surprising that *hsp90* exhibited different profiles at two different temperatures. The *ace* expression difference is more difficult to explain, given that it encodes a protein with neuronal function (32). Interestingly, as both *ace* and *hsp90* generated similar temperature-dependent profiles, it is possible that they are part of a gene expression module that responds to the temperature differences, although such a relationship has not been reported in the literature.

The temperature effect on *ace* expression (similar to those shown for *hsp90* in Fig. 2*b*) seemed to be counter to the ANOVA models, wherein no temperature effect was detected for that locus. This resulted from interaction among strain, temporal block, and environment, wherein replicates of the MI strain responded variably to low temperature treatments, as well as differently than the CA strain. The unique expression pattern exhibited by this locus is interesting for two reasons. First, even with the relatively high variance in expression that temperature and strain introduced, *ace* was still significantly informative of age, although it was clearly not the best predictor among loci tested. Second, its unique response to temperature raises the possibility of identifying populations based on their expression profiles, because the MI strain responded to low temperatures differently than the CA strain, as well as helping to uncover environmental factors that may affect plasticity in development. These observations indicate that loci like *ace* could potentially help decrease error through the identification of genotypes and environments that lead to variation in blow fly age estimates.

The utility of the models was then examined in a blind study, testing their ability to accurately and precisely predict immature blow fly development, and evaluating the nature of any error produced. The former simply tell us the overall utility of including gene expression data in age approximations. The latter is critical to consider for PMI estimates, given the legal ramifications. Underestimates of fly age are expected in natural conditions and thus are accounted for by forensic entomologists, who generally define a minimum PMI. Underestimates happen for two reasons: first, since many flies have the opportunity to oviposit on a body over the course of several days, there will be oviposition events that occur after the initial ones, thus many insects sampled from a body will not represent the oldest individuals. Second, even within a cohort of eggs laid at the same time, blow flies exhibit different development rates (5), with slower development likely resulting from individual exposures to localized environmental factors (22). In general then, it is more important to decrease overestimates of age, as underestimates are anticipated and therefore are not misleading.

Examination of accuracy, precision, and error in age estimation indicates that the results of this study were mixed (Fig. 6). The control model, incorporating developmental stage, length, weight, strain, and temperature, produced accurate but imprecise estimates. Type A models, which included only small amounts of gene expression information, improved the precision of larval age predictions slightly, while Type B predictions were accurate and showed improved precision for both larvae and pupae. The increase in precision in the blind study was *c.* 3–8% depending on the environment and type of model evaluated, and occurred by decreasing the level of overestimation. This pattern could be anticipated from the diagnostic plots for all models (Figs 3 and 4), given there was a bias in predicted error toward underestimates. As the blind study was conducted with single cohorts of eggs, multiple cohorts were not responsible for underestimates, leaving developmental variation (because of genetics or the environment) as the apparent cause. Likewise, models were developed from a data set wherein older individuals were preferentially sampled, which would also skew subsequent age predictions toward underestimates, assuming blind sampling represented average development of the cohort more than the most developed individuals.

Age estimates in the blind study, when cohorts were raised at the stable temperatures used to develop gene expression profiles, were accurate and precise for both larval and pupal samples. However, estimates of flies raised in ambient conditions resulted in a bias toward greater underestimation of fly development. *L. sericata* development has been shown to slow in variable temperatures (5), which may explain this result. The bias may also have been exaggerated by the fact that, while very strongly correlated, there was an observed difference between development percent as measured in hours at constant temperature versus development percent as measured in accumulated degrees. When correcting for this effect (see Results), the upper limit of the prediction range was within 1% of true development, although both larvae and pupae still yielded underestimates. Whatever the reason for the discrepancy between the two measures of developmental progress, the phenomenon must be more fully explored before gene expression analyses can be applied to ambient temperature predictions.

Models developed with length-anchored gene expression resulted in the lowest predicted error rates, yet yielded a higher error range than all other models when used in the blind study. There are two possible explanations for this. The models could have been too specific to the length data used to develop them, resulting in higher error when making predictions with another data set exhibiting different length characteristics. Alternatively, and more likely, the error was a result of converting preserved length to live length, because preservation resulted in shrinkage of larval samples. This conversion was carried out using regressions, with R^2 values that were lowest for postfeeding third instars and highest for feeding third instars. The conversion likely resulted in an inaccurate prediction of length, especially for postfeeding third instars, which would affect estimates made with models that anchored expression to length terms.

Conclusions

Current methods used to predict a PMI with blow fly evidence associate longer developmental stages with higher error. Incorporating additional information into the prediction framework has the potential to improve the precision of blow fly age estimates, and thus PMI estimates. The addition of gene expression data to more precisely predict blow fly development appears promising, although the effects of genotype and the environment, especially fluctuating environments, will need to be examined in greater detail. The benefit of genetic assessment of blow fly age is most pronounced during the postfeeding third instar and pupal stages, which produce the least precise estimates using traditional morphological data. Additionally, more continuous predictions of pupal age were made possible when expression data were included. The nine loci studied herein resulted in a relatively modest 3–8% decrease in age overestimation when evaluated in a blind study; however, this set of genes was chosen based on available sequence data and undoubtedly does not represent the most informative markers possible. Efforts to identify more developmentally informative blow fly genes should improve age estimates and provide even more accurate PMI estimates using entomological data.

Conflict of interest: The authors have no relevant conflicts of interest to declare.

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Disclaimer

Points of view in this document are those of the authors and do not necessarily represent the official position or policies of the U.S. Department of Justice.

References

1. Catts EP, Haskell NH. Entomology and death: a procedural guide. Clemson, SC: Joyce's Print Shop, Inc., 1990.
2. Greenberg B, Kunich JC. Entomology and the law flies as forensic indicators. Cambridge, UK: Cambridge University Press, 2002.
3. Grassberger M, Reiter C. Effect of temperature on *Lucilia sericata* (Diptera: Calliphoridae) development with special reference to the isomegalen- and isomorphen-diagram. Forensic Sci Int 2001;120:32–6.
4. Greenberg B. Flies as forensic indicators. J Med Entomol 1991;20:565–77.
5. Anderson GS. Minimum and maximum development rates of some forensically important Calliphoridae (Diptera). J Forensic Sci 2000; 45:824–32.
6. Wells JD, Kurahashi H. *Chrysomya megacephala* (Fabricius) (Diptera: Calliphoridae) development: rate, variation, and the implications for forensic entomology. Jpn J Sanit Zool 1994;45:303–9.
7. Wells JD, Lamotte LR. Estimating maggot age from weight using inverse prediction. J Forensic Sci 1995;40:585–90.
8. Tarone AM, Foran DR. Generalized additive models and *Lucilia sericata* growth: assessing confidence intervals and error rates in forensic entomology. J Forensic Sci 2008;53:942–8.
9. Kalthoff K. Analysis of biological development. New York, NY: McGraw-Hill, 2001.
10. Arbeitman MN, Furlong EEM, Imam F, Johnson E, Null BH, Baker BS, et al. Gene expression during the life cycle of *Drosophila melanogaster*. Science 2002;297:2270–5.
11. Beckstead RB, Lam G, Thummel CS. The genomic response to 20-hydroxyecdysone at the onset of Drosophila metamorphosis. Genome Biol 2005;6:R99.
12. McAlpine JF. Manual of nearctic diptera. Hull, Quebec: Canadian Government Publishing Center, 1989.
13. McGregor AP. How to get ahead: the origin, function, and evolution of *bicoid*. Bioessays 2005;27:904–13.
14. Ali RA, Mellenthin K, Fahmy K, Da Rocha S, Baumgartner S. Structural conservation of the salivary gland-specific slalom gene in the blowfly *Lucilia sericata*. Dev Genes Evol 2005;215:537–43.
15. Yeates DK, Weigmann BM. The evolutionary biology of flies. New York, NY: Columbia University Press, 2005.
16. Mellenthin K, Fahmy K, Ali RA, Hunding A, Da Rocha S, Baumgartner S. Wingless signaling in a large insect, the blowfly *Lucilia sericata*: a beautiful example of evolutionary developmental biology. Dev Dyn 2006;235:347–60.
17. Tarone AM, Jennings KC, Foran DR. Aging blow flies using gene expression: a feasibility study. J Forensic Sci 2007;52:1350–4.
18. Kamal AS. Comparative study of thirteen species of sarcosaprophagous Calliphoridae and Sarcophagidae (Diptera) I. Bionomics. Ann Entomol Soc Am 1958;51:261–70.
19. East IJ, Eisemann CH. Vaccination against *Lucilia cuprina*: the causative agent of sheep blowfly strike. Immunol Cell Biol 1993;71:453–62.
20. Hastie TJ, Tibshirani RJ. Generalized additive models. Boca Raton, FL: Chapman and Hall/CRC, 1990.
21. Wood SN. Generalized additive models: an introduction in R. Boca Raton, FL: Chapman and Hall/CRC Taylor and Francis Group, 2006.
22. Tarone AM, Foran DR. Components of plasticity in the development of a Michigan population of *Lucilia sericata*. J Med Ent 2006;43:1023–33.
23. Gibson G, Weir B. The quantitative genetics of transcription. Trends Genet 2005;21:616–23.
24. Mackay TFC. The genetic architecture of quantitative traits. Annu Rev Genet 2001;35:303–39.
25. Conner JK, Hartl DL. A primer of ecological genetics. Sunderland, MA: Sinauer Associates, Inc., 2004.
26. R Development Core Team. R: a language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing, 2004, ISBN 3-00051-00-3, http://www.R-project.org (accessed November 11, 2010).
27. Tarone AM. *Lucilia sericata* development: plasticity, population differences, and gene expression [dissertation]. East Lansing (MI): Michigan State Univ., 2007.
28. Foran DR. Generating more precise post mortem interval estimates with entomological evidence: reliable patterns of gene expression throughout Calliphorid larval and pupal development. East Lansing (MI): Michigan State Univ., 2007 Aug. Department of Justice Document No.: 219503.
29. Thummel CS. Ecdysone-regulated puff genes 2000. Insect Biochem Mol Biol 2002;32:113–20.
30. Sullivan AA, Thummel CS. Temporal profiles of nuclear receptor gene expression reveal coordinate transcriptional responses during *Drosophila* development. Mol Endocrinol 2003;11:2125–37.
31. Spradling A, Pardue ML, Penman S. Messenger RNA in heat-shocked *Drosophila* cells. J Mol Biol 1977;109:559–87.
32. Gnagney AL, Forte M, Rosenberry TL. Isolation and characterization of acetylcholinesterase from *Drosophila*. J Biol Chem 1987;262:13290–378.

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