Population and Temperature Effects on *Lucilia sericata* (Diptera: Calliphoridae) Body Size and Minimum Development Time

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ABSTRACT Understanding how ecological conditions influence physiological responses is fundamental to forensic entomology. When determining the minimum postmortem interval with blow fly evidence in forensic investigations, using a reliable and accurate model of development is integral. Many published studies vary in results, source populations, and experimental designs. Accordingly, disentangling genetic causes of developmental variation from environmental causes is difficult. This study determined the minimum time of development and pupal sizes of three populations of *Lucilia sericata* Meigen (Diptera: Calliphoridae; from California, Michigan, and West Virginia) at two temperatures (20°C and 33.5°C). Development times differed significantly between strain and temperature. In addition, California pupae were the largest and fastest developing at 20°C, but at 33.5°C, though they still maintained their rank in size among the three populations, they were the slowest to develop. These results indicate a need to account for genetic differences in development, and genetic variation in environmental responses, when estimating a postmortem interval with entomological data.

KEY WORDS *Lucilia sericata*, Calliphoridae, forensic entomology, life history, ecological genetics

Understanding how ecological conditions drive the evolution of body size and development time is a fundamental theme in biology (Oudman et al. 1991, Kingsolver 2000, Morey and Reznick 2000, Day and Rowe 2002, Hallas et al. 2002, Feder et al. 2003, Plaistow et al. 2004, Gomez-Mestre and Buchholz 2006). This has led to research into how resource-limited organisms, particularly those that undergo a complete developmental metamorphism, leverage a trade-off between developing quickly (i.e., ensuring survival to adulthood) and producing larger, more fit adults by delaying maturation (Wilbur and Collins 1973). An oft-studied example is juvenile spadefoot toads, which develop in desert vernal ponds. Some spadefoot species have evolved to delay development under low resource conditions, whereas others continue to develop while sacrificing body size, and thus reproductive success (Morey and Reznick 2000, Gomez-Mestre and Buchholz 2006). However, attempts to model such trade-offs have only been partially successful, in that some species produce the largest individuals when growth is at its fastest. This has led to the hypothesis that developmental thresholds (minimum requirements for life history events to occur) play a role in physiological processes that determine body size and development time, influencing relationships between size and age at maturity (Day and Rowe 2002). The presence of developmental thresholds has been partially confirmed in the mite species *Scancassania berlesei* (Michael) (Acari: Acaridae), which demonstrated that the predicted shift in size reaction norms was regulated by growth conditions, sex, and maternal nutrition (Plaistow et al. 2004). Furthermore, in *Drosophila* (Diptera: Drosophilidae) and other holometabolous insects, there are well-known developmental thresholds, such as minimum viable weight and critical weight, which are important in the balance between size and age at maturity (Mirth and Riddiford 2007). Additionally, performance curves of the butterfly *Pieris rapae* (Lepidoptera: Pieridae) indicate that the evolution of life history traits is influenced by selection pressures on developmental responses to temperature (Kingsolver 2000, Kingsolver et al. 2001). Taken together, these data highlight the influence of physiological attributes on the evolution of life history traits.

One key feature of organisms that experience irregular resources leading to development trade-offs between body size and development time is the evolution of phenotypic plasticity (environmentally induced changes in phenotypes) (Day and Rowe 2002). Blow flies (Diptera: Calliphoridae) depend on ephemeral food supplies (carrion and biological waste) for

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development, which might make such plasticity advantageous, and has been demonstrated in the blow fly *Lucilia sericata* Meigen, wherein size and age at maturity are responsive to changes in laboratory-rearing conditions such as moisture and type of food (Clark et al. 2006, Tarone and Foran 2006). Furthermore, the global distribution of some Calliphoridae species could result in selection for variable body sizes and development times among populations. Finally, differences in selection pressures might lead to genetic variation in environmental responses, which has not been examined in blow flies.

Identifying factors that influence blow fly development has applied uses. First, calliphorids cause myiasis in humans (Faulde et al. 2001), sheep (East and Eisemann 1993), and cattle (Crystal and Ramirez 1975). Blow fly development is also used as a biological clock to help estimate a minimum postmortem interval (PMI) in death investigations (Greenberg and Kunich 2002, Haskell and Williams 2008, Byrd and Castner 2010, Tomberlin et al. 2011a). Both myiasis and PMI predictions are associated with blow fly development; thus, understanding its variation should aid our ability to improve PMI predictions and limit the damage caused by myiasis. Different authors have, however, reported varying minimum development times within and among blow fly species (Kamal 1958, Greenberg 1991, Anderson 2000, Grassberger and Reiter 2001). This may result from disparity in laboratory-rearing conditions (Clark et al. 2006, Tarone and Foran 2006), but could also be affected by genetic differences. For instance, Greenberg (1991) noted variability in development times between Russian and United States *L. sericata*, although specific details were lacking. Gallagher et al. (2010) found significant differences in minimum times required to reach the postfeeding third instar stage at three rearing temperatures for *L. sericata* collected from San Diego and Sacramento, California, and Eaton, Massachusetts. Similarly, gene expression has been shown to be variable depending on temperature and strain effects (Tarone and Foran 2011), further supporting the idea that different strains of *L. sericata* exhibit variable developmental patterns. Indeed, variation in body size and development time (the core phenotypes of interest to forensic entomologists) has been observed in multiple Cyclorrhaphan species (reviewed in Tarone and Foran 2006, Tomberlin et al. 2011a).

If minimum development times are the same across populations, age predictions do not need to be calibrated among them. However, if development times vary, age estimates will differ, requiring standardization appropriate to the flies of interest. This poses a problem to the forensic science community, as it would necessitate either a database of development times for any region where forensic entomology is employed, post priori studies to determine the specific minimum development time of the population of interest, or a detailed understanding of factors predictive of developmental differences. The research presented here was designed to test the hypothesis that genotype, the environment, and the interaction of the two affect development time and body size in *L. sericata.* Differences among strains may result from random drift among populations (Picard and Wells 2010), or alternatively, selection may drive *L. sericata* populations toward different development times and body sizes, independent of neutral genetic divergence. An understanding of how size and age at maturity are physiologically regulated, as well as the ecological forces that might lead to their evolution (Tomberlin et al. 2011b), will provide a more detailed appreciation of factors influencing errors in entomologically derived estimates of PMI.

Materials and Methods

L. sericata were collected in the summer of 2006 from Davis, California (CA); East Lansing, Michigan (MI); and Morgantown, West Virginia (WV). Rearing conditions and species identifications were based on morphological and genetic criteria (*cytochrome oxidase 1* sequences), as previously described (Tarone and Foran 2006). Ten cohorts of each strain were induced to oviposit and assigned to a temporal block composed of all flies from a strain laid on that day. Cohorts were split between 20.0°C and 33.5°C temperature treatments, and the lengths (to the nearest 0.5 mm) and weights (to the nearest 0.01 mg) of 12 individuals were measured on the third day of pupation. Likewise, the minimum development time (to the nearest 0.5 h) for each cohort was recorded and converted to accumulated degree hours using 6°C as the minimum threshold temperature (Byrd and Castner 2010).

This scheme resulted in 120 fly length and weight measurements for each strain at each temperature. Mean, median, minimum, and maximum values were analyzed through a two-way analysis of variance (ANOVA) model assessing all potential interactions, using the software package JMP (JMP 2009). Ranges for development time reflect minimum, average, and maximum observed minimum development times. The analysis of development time was first conducted singly with temporal block and replicate set as random variables given limited degrees of freedom. These effects were not significant; thus, a two-way ANOVA was conducted, excluding them and including the interaction between strain and temperature. A post hoc *t* test, assuming heteroscedastic data, was performed on significant findings to determine if any paired analyses were themselves significantly different. Pupal weight and length were analyzed using a nested model (replicates nested within strains nested within blocks nested within temperatures), again followed by a post hoc *t* test. Significant *P* values between 0.05 and 0.0083 (for minimum development time) or 0.0072 (for weight and length) should be considered with caution as multiple comparisons were included.

Table 1. Phenotype summaries for 10 replicates of three regional *L. sericata* strains originating from Davis, California (CA); East Lansing, Michigan (MI); and Morgantown, West Virginia (WV)

Strain	Phenotype	Temp (°C)	*N*	Min. (ADH)	Max (ADH)	Mean (ADH)	Median (ADH)
CA	Min. dev. time	20	10	431.5 (6,041)	497.5 (6,965)	458.9 (6,425)	455.5 (6,377)
MI	Min. dev. time	20	10	430 (6,020)	548 (7,672)	463.9 (6,495)	454.5 (6,363)
WV	Min. dev. time	20	10	428 (5,992)	572 (8,008)	475.5 (6,657)	455.5 (6,377)
CA	Min. dev. time	33.5	10	264 (7,260)	357.5 (9,831)	308 (8,470)	312.3 (8,587)
MI	Min. dev. time	33.5	10	237 (6,518)	311 (8,553)	276.2 (7,594)	278.8 (7,666)
WV	Min. dev. time	33.5	10	260.5 (7,164)	333 (9,158)	282.1 (7,757)	274.3 (7,542)
CA	Pupal length	20	120	7.5	9.0	8.23	8.0
MI	Pupal length	20	120	7.0	9.5	7.87	8.0
WV	Pupal length	20	120	6.5	8.5	7.63	7.5
CA	Pupal length	33.5	120	6.5	9.0	7.41	7.5
MI	Pupal length	33.5	120	6.0	9.0	7.30	7.5
WV	Pupal length	33.5	120	6.0	8.0	6.98	7.0
CA	Pupal weight	20	120	36.27	63.03	44.80	44.77
MI	Pupal weight	20	120	26.86	49.86	38.33	37.33
WV	Pupal weight	20	120	19.18	48.25	34.90	34.66
CA	Pupal weight	33.5	120	13.01	42.28	28.63	29.04
MI	Pupal weight	33.5	120	14.10	40.11	28.16	27.6
WV	Pupal weight	33.5	120	16.22	37.86	25.08	24.81

ADH, accumulated degree hours; Min. dev., minimum development. Minimum development time statistics are reported in hours with accumulated degree hours in parentheses. Pupal weight is reported in mg, and pupal length in mm.

Results

All three fly strains were positively identified as *L. sericata.* A 616-bp sequence from the CA strain (accession GU395205) was 100% concordant with published *L. sericata* sequences. A 686-bp sequence from the MI strain (accession EU848424) was 100% concordant with published *L. sericata* sequence. A 776-bp sequence from the WV strain (accession GU395206) was 100% concordant with published *L. sericata* sequence. In all cases, no other species had this level of sequence identity.

Minimum, maximum, and average minimum developmental times, along with pupal lengths and weights, are shown in Table 1 for each strain and environment. Findings were the same regardless if data were analyzed as absolute development time or accumulated degree days.

Development times varied among the strains and between the temperatures. At 20°C, CA had the shortest developmental time, followed by MI and WV (Fig. 1). In contrast, CA had the longest developmental time at 33.5°C, followed by WV and MI. A two-way ANOVA of minimum development times demonstrated significant differences among strains and temperatures (Table 2). The interaction of temperature with strain was not significant, albeit a moderately low *P* value was obtained (Table 2). Development times at the two temperatures were significantly different for all three strains, with overall development taking longer at the lower temperature (*t* test, $P < 0.01$). There were no significant differences in minimum development times among the strains at 20°C (*t* test, $P > 0.30$ for all), but at 33.5°C, flies originating from CA exhibited marginally slower development rates than flies originating from either MI or WV (Fig. 1, $P = 0.01$ and $P = 0.05$, respectively), indicating an interaction between the CA genotype and temperature for this phenotype.

There were also significant effects of strain and temperature on pupal weight and length with nested ANOVA models (replicates nested within strain nested within blocks nested within temper-

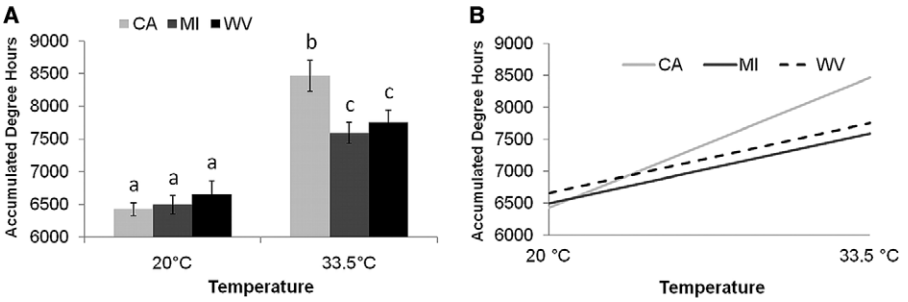

Fig. 1. Mean minimum developmental times ± SE in accumulated degree hours (using a minimum threshold temperature of 6°C) for the three stains (CA, MI, and WV) reared at two different temperatures (20°C and 33.5°C). (A) Comparisons between strains and the two temperatures are shown, with different letters representing comparisons distinguishable by $P \leq 0.05$. Error bars represent standard error. (B) Reaction norm plot for the minimum development time for the three strains at the two temperatures.

Table 2. ANOVA table for minimum development time, pupal length, and weight

Phenotype	Model	Source	df	Mean square	*F* ratio	$P > F$
Minimum development time	Two-way ANOVA	Model	5	6,929,419	17.7161	<0.0001
		Error	54	391,136		
		Total	59			
	Effect tests	Temperature	1	30,023,294	76.7591	<0.0001
		Strain	2	820,511	3.8130	0.0282
		TempXStrain	2	1,491,391	2.0978	0.1326
Pupal length	Nested ANOVA	Model	59	3.26560	21.4680	<0.0001
		Error	660	0.15211		
		Total	719			
	Effect tests	Replicate	3	0.76549	5.0324	0.0019
		Strain (rep)	5	7.47399	49.1339	<0.0001
		Block (strain, rep)	21	1.93221	12.7023	<0.0001
		Temperature (block, strain, rep)	30	3.7843	24.8784	<0.0001
Pupal weight	Nested ANOVA	Model	59	779.983	42.3969	<0.0001
		Error	660	18.397		
		Total	719			
	Effect tests	Replicate	3	14.985	0.8145	0.4861
		Strain (rep)	5	1,136.215	61.7603	<0.0001
		Block (strain, rep)	21	237.275	12.8974	<0.0001
		Temperature (block, strain, rep)	30	1,145.372	62.2581	<0.0001

atures; Table 2, Fig. 2). Multiple pairwise comparisons of pupal lengths and weights showed that they were significantly different within strains at the two temperatures (*t* test, all $P \leq 0.001$) as well as between pairs of strains at both temperatures (all $P \leq 0.001$). An exception was the CA and MI strains reared at 33.5°C, which did not differ significantly in pupal length or weight (*t* test, $P = 0.15$ and $P = 0.55$, respectively).

Discussion

The research presented here uncovered several interesting factors that help shed light on the evolution of *L. sericata* age and size at metamorphosis. First, pupal size differed among genotypes and between temperatures; the CA strain was consistently the largest, whereas WV was smallest, and in all instances lower rearing temperatures resulted in larger pupae. Second, there was no straightforward relationship between body size and development time: at 20°C the smallest strain (WV) required the longest time to reach eclosion, and the largest (CA) matured in the least amount of time. Day and Rowe (2002) noted that the largest individuals of a species generally develop the fastest. They hypothesized that this could be explained by thresholds regulating developmental progression, an idea that is well supported in flies (Mirth

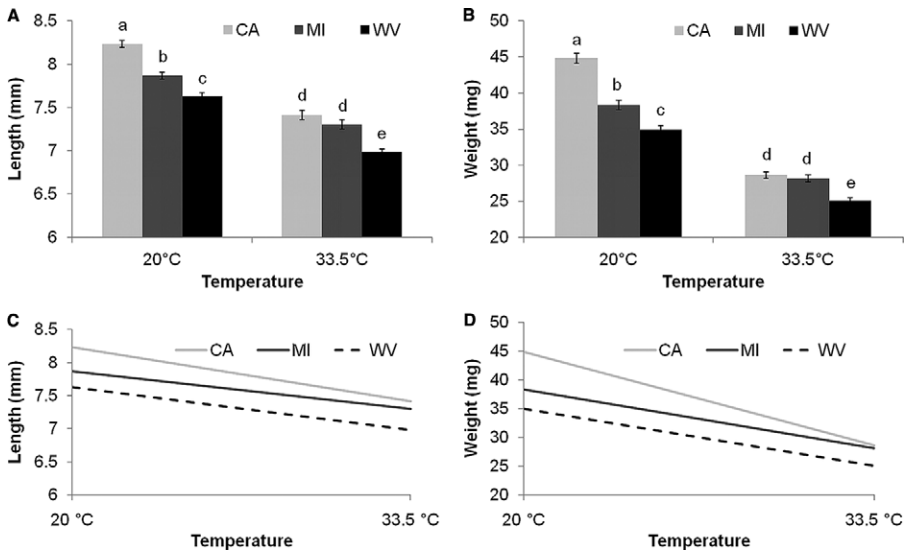

Fig. 2. Mean pupal length (A) and weight (B) ± SE for three strains (CA, MI, and WV) at two rearing temperatures (20°C and 33.5°C). Comparisons between different columns are shown, with the different letters (a–e) representing a significant difference ($P \leq 0.001$). Error bars represent standard error. (C) and (D) depict the reaction norms for the three strains at the two temperatures for length and weight, respectively.

and Riddiford 2007). The 20°C developmental data of the flies studied here would support the hypothesis that developmental thresholds exist in *L. sericata* and are involved in the evolution of these strains. However, at 33.5°C, MI flies developed fastest, whereas CA flies took the longest to mature. The change in rank order of minimum development times suggests that developmental thresholds may not be the target of selection, are not the only target of selection, or that multiple thresholds influence blow fly development. The relative differences in development patterns among strains and between temperatures indicate the ecosystems from which the strains originated push them toward different development optima, reminiscent of alternative optimal developmental temperatures for caterpillar life history traits (Kingsolver 2000, Kingsolver et al. 2001).

Ecological differentiation among the fly strains examined in this study seems plausible given the dissimilarities in the ecoregions (distinct ecosystems as defined by a multivariate analysis of biotic and abiotic components of different United States regions; Hargrove and Hoffman 2004) from which each strain originated. Specific knowledge of ecological factors that drive the evolution of physiologically important genes would be useful in dissecting the root causes of such differentiation and in confirming whether it results from evolution of genes influencing developmental thresholds like critical weight. In this regard, numerous factors have already been identified that can influence the evolution of fly physiology. For example, in Calliphoridae, latitude correlates with upper temperature thresholds during development (Richards et al. 2009). Likewise, body size and development time in *Drosophila* spp. (Diptera: Drosophilidae) (Oudman et al. 1991, Hallas et al. 2002) and *Rhagoletis pomonella* (Diptera: Tephritidae) (Feder et al. 2003) also correlate with latitude. Pupal diapause varies with geographic origin in Asian and Oceanic *Boettchersca* spp. (Diptera: Sarcophagidae) (Kurahashi and Ohtaki 1989). Various ecological factors (e.g., elevation, light, temperature, moisture, the presence of different species) that vary among regions could contribute to the evolution of those traits within a population. In this study, the development times of the MI and WV strains responded similarly to thermal shifts and differed considerably from the thermal response of the CA strain (Fig. 1). Any environmental factors shared between MI and WV, but not with CA (e.g., prolonged exposure to freezing temperatures), could result in differential selection on thermal plasticity in development time.

Alternatively, studies of the blow flies *Phormia regina* Meigen and *L. sericata* indicate that individuals collected around the same time are likely to be related (Picard and Wells 2009, 2010). If this were the case in the current research, differences in phenotypes among strains may be random in nature, not systematic. However, earlier examinations of the fly populations studied here (Tarone and Foran 2008) showed the same developmental trends, even though they were collected 1 yr earlier, indicating that the size variation among strains was not random. Based on the numerous environmental features known to result in life history trait evolution in other fly species, and the lack of evidence for random effects, the size and developmental time plasticity presented in this work are most likely the result of differential selection among the populations studied.

Two factors could readily explain the temperature/size relationship found in *L. sericata.* First, development is dependent on metabolism and the mechanical acquisition of food. If high temperatures result in feeding rates that do not keep up with increased metabolism, then flies raised at higher temperatures would be expected to develop into smaller adults (Perrin 1995, Atkinson 1996). In *L. sericata*, increased growth rates at higher temperatures have been noted (Tarone and Foran 2008), indicating that a disproportionately greater increase in metabolism may have occurred. Second, increased mortality at high temperatures has been proposed as a mechanism for promoting smaller sizes in high temperature environments (Berrigan and Charnov 1994, Sibly and Atkinson 1994). Essentially, if larvae are more likely to die in warmer conditions because of factors such as heat stress or anoxia, there may be selection to complete development quickly at the expense of body size. Although mortality was not officially recorded in the current study, it was observed that there was noticeably higher mortality at 33.5°C, especially in the smallest strain (WV), which supports this theory. Given these results, it seems likely that factors such as heat stress and anoxia are helping to drive the evolution of small body sizes at high temperatures in these organisms.

An explanation for temperature and strain influences on body size, development time, and gene expression levels can be found in quantitative genetic theory (reviewed in Conner and Hartl 2004). Any continuously variable phenotype is subject to the influence of genetics, the environment, and the interaction between the two, which can be detected through reaction norms and ANOVA (Scheiner and Gurevitch 2001, Conner and Hartl 2004). Entomologists use the quantitative traits of development time, length, and weight for blow fly age predictions (Kamal 1958, Greenberg 1991, Anderson 2000, Grassberger and Reiter 2001); therefore, phenotypes can be studied with a quantitative genetic approach to understand if and how environment and genotype affect them. Knowledge of such factors will help increase the accuracy of blow fly age predictions by identifying variables that affect the maturation of the Calliphoridae.

These findings have clear consequences for the field of forensic entomology. There are inherent inaccuracies that exist when predicting a PMI with blow fly evidence, which are rooted in population/quantitative genetics and stem from the fact that phenotypes can vary among blow fly strains and environments (Tarone and Foran 2008, Gallagher et al. 2010, Tomberlin et al. 2011a). This can lead to poor PMI estimates if the development of a specific strain is not in accordance with the data used to make age predic-

tions. For example, a precise prediction that an individual has completed 70% of its development to adulthood does not necessarily represent the same amount of time in two different populations; if one population develops in 500 h at a given temperature, whereas another develops in 470 h, 70% would correspond to ages of 350 and 329 h, respectively. The occurrence of strain effects could necessitate population-specific data to calibrate PMI estimates. Unfortunately, this problem is further exasperated by the genotype-environment interactions in size and development time (the major phenotypes associated with forensic entomology) reported here, which indicate that a universal adjustment among strains is not possible, as each can respond differently to thermal changes.

This challenge might be mitigated in part by considering evolutionary ecology theory (Tomberlin et al. 2011b), which seeks to explain phenotypic variation in terms of ecological effects (reviewed in Conner and Hartl 2004). Guiding principles behind the variation in development times in *L. sericata* and other forensically informative arthropods can most easily be elucidated by studying a series of populations, whereas ignoring population differences could lead to inaccurate PMI predictions based on single populations. One possible short-term method for developing a population-specific PMI would be to calibrate development times in published data with population-specific developmental information (e.g., is the population a slower developing population?), thereby developing a posterior blow fly age estimate tailored to the population of interest (see Scheiner and Gurevitch 2001). Clearly, it is impossible to determine the development times of all populations of blow flies in all environments, but it may be feasible to understand the factors influencing the evolution of forensically important fly traits. In the long-term, factors that affect, or are predictive of, forensically informative blow fly phenotypes will need to be incorporated into PMI estimates. For example, if flies develop more quickly at higher elevations than lower ones, integration of elevation data (including elevation-associated genetic polymorphisms) into PMI predictions could provide more accurate PMI estimates without requiring specific knowledge of the population in question. Elucidating such variables and incorporating them into PMI estimates will decrease error in age predictions made for fly populations that differ from laboratory-based tables of development times. The identification of factors influencing developmental variation should continue to be a priority in forensic entomology research.

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