A GENETIC STUDY OF THE DEVELOPMENT OF *Cochliomyia macellaria* (FABRICIUS) (DIPTERA: CALLIPHORIDAE): ECOLOGICAL, EVOLUTIONARY, AND FORENSIC IMPORTANCE OF THE SECONDARY SCREWWORM

A Thesis

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
In partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

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August 2015

Major Subject: Entomology

ABSTRACT

Blow flies (Diptera: Calliphoridae) are carrion feeding insects that perform a critical ecological service by recycling nutrients throughout temperate and tropic regions of the world. Some members of this guild are parasites of vertebrates and important to medical and veterinary research. Given their relatively predictable life cycles and close association with carrion, estimating blow fly age from human and animal remains can also be informative in legal investigations. In the southern United States, the blow fly *Cochliomyia macellaria* (Fabricius), known as the secondary screwworm, is a common primary colonizer of carrion, can parasitize mammals, and often is used as evidence in forensic investigations. Accordingly, studies of this organism can contribute to our basic knowledge of decomposition ecology. Life history traits are important to the evolutionary fitness of any organism and yet, there are very few studies on genetic variation of these phenotypes in blow flies even though knowledge of that variation would aid in medical, agricultural, and forensic endeavors.

Natural genetic variation in development time and body size has been observed in *C. macellaria*. To further evaluate genetic variation in immature development rates of this species, an artificial selection experiment was performed on three Texas populations. After 23 generations of selection at 25°C, all experimental populations selected for faster development exhibited approximately a 1.5 days decrease in mean development times as compared to the founding generation, while those bred for slow

development required another 3.5 days of development. The six selected lines were subsequently reared in different thermal environments (20°C, 25°C, and 30°C), to evaluate their phenotypic plasticity in development time, pupal mass, and immature viability. Under different thermal exposures, *C. macellaria* developed slower at cooler temperatures and faster at warmer temperatures with a difference between selection groups of approximately 3.7 days at 30°C, 5.6 days at 25°C, and 7.9 days at 20°C. Slow developers reared at 25°C and 30°C had significantly heavier pupal mass (48.38-51.85 mg) than the cooler and slower selected lines (38.00-40.99 mg). Immature viability also differed between selection regimes, with survival to adulthood ranging from 54.8-86.7% for all blow flies studied here. Significantly higher lethality was observed in the slow developing blow flies, especially at cooler temperatures. The results of this work suggest that *Cochliomyia macellaria* harbor considerable genetic variation for development time, are more likely to be faster developing in the wild, and that alleles that slow development alter correlations among life history traits in a temperature dependent manner. This study of the natural variation in basic blow fly biology provides valuable information regarding the evolutionary biology and ecology of the species. The selected lines provide the basic material to conduct further genetic studies to identify markers associated with variation in development time. These markers could ultimately be used to improve accuracy in estimating the ages of immature secondary screwworms for forensic purposes.

DEDICATION

This thesis is dedicated to my family with much love, to truly let me develop as a scientist.

ACKNOWLEDGMENTS

I would like to acknowledge my loving family. Life and school would have been much harder without their support. I would also like them for stomaching my occasional bug stories, regardless of how cool or gross the stories were.

I need to thank my employers at the Image Analysis Laboratory, Dr. Robert C. Burghardt and Dr. Rola Barhoumi and staff. With their support to continuing my education, I was able to pursue a master of science.

I would like to thank my committee for their valued input and patience as I pursued a master's degree while working full time. Dr. Aaron Tarone, Dr. Jeffery Tomberlin, Dr. Spencer Johnston, and Dr. Clifford Spiegelman help expand my horizons and learn new perspectives on my scientific research. Thank you.

Thanks to the assorted F.L.I.E.S. Facility members: Rachel Mohr, Charity Owings, Micah Flores, Meaghan Pimsler, Adrienne Brundage, Ashleigh Faris, and Jonathan Parrott. They were of great help, offering excellent feedback, and brought some excitement into the lab in the monotony of me counting thousands, upon thousands of flies.

I would like to thank the professors Dr. Robert Wharton, Dr. James Woolley, and Dr. Roger Gold, who all helped solidify my interest in entomology. The spark of interest in insects, on top of my background in genetics, helped me become a better scientist.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Blow flies (Diptera: Calliphoridae) are important ecologically and contribute to numerous areas of biological research. Many of these flies perform a critical ecosystem service by functioning as decomposers of vertebrate remains, serving as a means for dispersing nutrients from the remains back to the ecosystem. This ecological function means blow flies can provide information towards a death investigation, and inform forensic entomology research, that allows death investigators to infer a post-mortem interval (PMI, Erzinclioglu 1983, Byrd and Castner 2001). Some species of blow flies are parasites of vertebrates, engaging in a behavior known as myiasis, whereby larvae infest and feed on living tissue. Myiasis can have both positive and negative impacts in medical and veterinary research. Negative impacts of myiasis can be extensive and result in large monetary losses, for instance in the wool industry in Australia. Such economic damage resulted in the development of the sterile insect technique (S.I.T.) to remove the primary screwworm from North America (Vargas- Terán et al. 2005, Mastrangelo and Welch 2012). Blow flies also cause myiasis of humans (Batista-da-Silva 2011). However, myiasis by some species can be beneficial and is a focal point of research directed toward improving wound healing (Mumcuoglu et al. 1999, Sherman 2003, Mumcuoglu 2007) and identifying novel antibiotics (Horobin et al. 2005). Finally, a

number of blow flies act as disease vectors, transferring pathogens among humans and animals (Zumpt 1965, Greenberg 1971, Maldonado and Centeno 2003, Stevens 2003, Heath and Bishop 2006). Because many of the important aspects of applied blow fly biology are centered on immature biology, more research needs to be done to understand immature blow fly biology and related components of evolutionary fitness.

Practical genetic research on the development of Calliphoridae is underrepresented in entomological literature, but that is steadily improving. In other model species, genomic tools have been devised to identify the genetic components of a broad range of biological problems. These endeavors can range from differentiating human (*Homo sapiens*) populations through SNP genotyping (Olshen et al. 2008) or identifying regions of the genome of *Anopheles gambiae* (Giles) (Diptera: Culicidae) that lead to reproductively isolated forms (Turner et al. 2005) or determining portions of the *Drosophila* genome that differentiated in size in selected experimental populations (Turner et al. 2011). Research using similar genomic tools could be directed to blow fly studies to identify markers of important life history traits, such as development rate and size, which are indicative of age in forensic entomology and important determinants of evolutionary fitness in many organisms. Recently, there has been a proliferation of basic research on the development of these important flies, which has implications for the positive and negative impacts of blow flies. In forensic entomology applied functional genomic research is being used to improve blow fly age estimates (Tarone et al. 2007, Tarone and Foran 2011, Sze et al. 2012) and expand our knowledge on conspecific genetic differences in life history traits of a species (Tarone et al. 2011, Owings et al.

2014). In medicine there has been a proliferation of molecular genetic and genomic studies targeted at understanding the molecular processes that make blow flies useful in maggot therapy (Otranto and Stevens 2002, Cazander et al. 2013). Further, while research examining the genetic mechanisms governing arthropod development traditionally has utilized model organisms such as *Drosophila melanogaster* (Meigen) (Diptera: Drosophilidae) and *Manduca sexta* (Linnaeus) (Hymenoptera: Sphingidae), these studies suggest that blow fly studies of the same nature could greatly improve our application of knowledge centered on blow fly biology. This thesis builds a foundation for such studies by addressing questions regarding the evolutionary ecology of *Cochliomyia macellaria* (Fabricius) (Diptera: Calliphoridae) and the genetics of its life history traits.

Cochliomyia macellaria thrive in the southern USA during the warmer months. Often found in association with animals and humans, its larvae habitually associate with decomposing remains of both. Consequently, it is a common pest and forensic indicator species. As noted above, a better understanding of its life cycle and developmental potential would help limit its damage and increase its utility in other fields, such as decomposition ecology and forensic entomology (Tomberlin et al. 2011b). Blow flies are carrion feeders, waiting for ephemeral resources to become available, then processing decisions on where and how long to search efficiently for carrion (Barton and Hovestadt 2013). Carrion provides a protein source to prepare females for ovary maturation, and a nutritional resource for the next generation. As *C. macellaria* are primary colonizers in the southern United States in the warmer months (Tomberlin and Adler 1998), they have

the advantage of gaining initial access to the decomposing organism and oviposit their eggs before other scavengers, predators, and competitors begin to consume the remains (Campobasso et al. 2001). The decomposing biomass quickly drops and immature blow flies likely make phenotypic trade-offs for their survival (Payne 1965, Pechal et al. 2013). Other consumers of ephemeral resources are known to make such trade-offs (Roff 1992, Davidowitz et al. 2005). For instance some organisms may delay development as an ephemeral resource depletes, sacrificing adult size, which can negatively impact reproductive fitness and dispersal capabilities. On decomposing remains, larvae compete for resources and avoid predators. One example of predation pressure comes from immatures of the Calliphorid *Chrysomya rufifacies* (Macquart) that predate on *C. macellaria* larvae (Wells and Greenberg 1992). Given that immature *C. rufifacies* can be facultative predators of immature *C. macellaria* (Wells and Greenberg 1992), this interaction likely puts selective pressures to develop faster and preempt predation. This is supported by the observation of priority effects (when a species' initial presence impacts subsequent community assembly patterns) when both of these species colonize resources together (Brundage 2011). Thus, immatures of *C. macellaria* face competing needs; the need to grow into a large, reproductively successful adult and the need to avoid predation and starvation to survive until adulthood before the resource is depleted. These competing needs are highly indicative of the potential for life history trade-offs, though little work on the topic exists in the carrion system.

Life history traits are often considered major factors of evolutionary fitness. The success of many species is dependent on genetic strategies for life history trait trade-offs

that allow them to handle and overcome biotic or abiotic challenges encountered as an organism develops and as a species adapts to their environment (Hoffmann and Parsons 1991). These trade-offs exist because it is very difficult for organisms to optimize all components of fitness at once. For instance, there are well-established trade-offs between reproduction and longevity (Luckinbill and Clare 1985), and reproduction and immunity (Luong and Polak 2007). One well-supported area of research in this field focuses on immature spadefoot toads that live in vernal ponds found in deserts. Eggs are laid when rain produces short-lived vernal ponds and species must adjust their development in the face of diminishing resources. When resources are scarce some tadpoles wait to develop, risking death if the pond dries up, in exchange for the chance to be a large adult, while others tadpoles make the trade-off for a smaller body size, which limits future mating options (Morey and Reznick 2000, Gomez-Mestre and Buchholz 2006). In addition to limiting mating options, small size can affect fecundity, as seen in Lepidoptera (Kozlowski 1992). This limitation is due to the fecundity-body size relationship that determines the number of viable offspring a female can produce. Such trade-offs can result in differences among species and populations of species that encounter divergent selection pressures. Among grasshoppers in California, embryonic development time and timing of embryonic diapause differs significantly among populations (Dingle and Mousseau 1994). More than 70% of grasshoppers from cooler environments developed quicker, while less than 26% of those from warmer habitats diapaused in the early stages of embryogenesis. Populations from cooler habitats took five days to complete embryonic development before hatching, while populations from

warm habitats required up to 20 days to hatch at 27°C. In the model insect *D. melanogaster*, a number of developmental differences have been noted, including differences in body size (Partridge et al. 1994, Turner et al. 2011) and development time (Prasad et al. 2000, James et al. 1995). Similar research documenting conspecific population differences has also been done for body size and development of *Lucilia sericata* (Meigen) (Diptera: Calliphoridae) (Tarone and Foran 2008, Gallagher et al. 2010, Tarone et al. 2011), *Drosophila pseudoobscura* (Frolova and Astaurov) (Diptera: Drosophilidae) (Dobzhansky and Levene 1951), *Manduca sexta* (Davidowitz et al. 2003), and the tortricids *Choristoneura spp.* (Lepidoptera: Tortricidae) (Schmidt and Lauer 1977). Taken together, conspecific population differences demonstrate conspecific variation in life history traits that are indicative of local adaptation (Conner and Hartl 2004). Some of these differences may represent different trade-off strategies employed by specific populations and those organisms may manage these strategies differently at a genetic level.

Complex phenotypes, such as development speed, are determined by genetic variation, environmental factors, and the interactions between them. These factors, in various assortments, can result in conspecific differences in such phenotypes. Allelic differences can be due to natural selection to survive in specific environments (Tarone et al. 2011). They can also be impacted by the environment and genetic drift of local wild populations, including the natural genetic variation present within the population (Picard and Wells 2010). The genetic capacity to display alternative morphological, physiological, and behavioral traits in response to the environment is known as

phenotypic plasticity (Pigliucci 2001, Garland, Jr and Kelly 2006, West-Eberhard 2003). This capacity can lead to different phenotypes in different environments, such as the well-known response of blow fly development to temperature, where higher temperatures generally lead to faster development rates. Genotype-by-environment interactions (GxE) demonstrate how different genotypes can differentially respond to the environment; this interaction is considered an indication of local adaptation if the affected phenotype influences fitness (Karban and Baldwin 1997, Conner and Hartl 2004).

There are a number of experimental approaches to evaluating the influences of genetics and the environment on complex fitness traits. Some routine approaches are common garden and reaction norm experiments. In a common garden experiment, also known as a transplant experiment, individuals of different populations are raised together in the same natural environment to test for genetic differentiation of a phenotypic trait or traits. In one common garden experiment, Glanville fritillary butterfly (*Melitaea cinxia* (Linnaeus) (Lepidoptera: Nymphalidae)) larvae were examined after winter diapause to observe their development. The caterpillars underwent an additional larval instar to prolong their development as an adaptation to the cooler environment. While this increased their size, the adaptation was more common in females (42%) than males (7%), and because of the delayed eclosion, males that did not adapt had a reduction in mating success due to their smaller size (Saastamoinen et al. 2013). Reaction norm experiments defined here as laboratory versions of common garden experiments, can also demonstrate how phenotypes are produced by genotypes in

different environments. This approach can also be valuable in understanding plasticity and genotype-by-environment interactions (Conner and Hartl 2004). For example, a reaction norm experiment tested thermal plasticity on *D. melanogaster*, to evaluate sexual dimorphism and genetic correlation at seven different temperatures. By exploring the natural body size variation of male and female fruit flies over a thermal spectrum, the study revealed that males and females do not differ much in size at lower temperatures (David et al. 2011).

Some reaction norm studies have been done with carrion feeding blow flies. An investigation on developmental variation of *L. sericata* reared at 20°C and 33.5°C showed that three populations collected from different states: Michigan, California, and West Virginia; exhibited a significant difference in their development times at the two temperatures. In this study, pupal weight and length were also measured and the California strain in that study was both the largest across both temperatures and the slowest developing at 33.5°C. Similarly, Gallagher et al. (2010) found significant difference in *L. sericata* development times at 16°C, 26°C, and 36°C among reared populations from Sacramento, CA, San Diego, CA, and Boston, MA. Research (Owings et al. 2014) on *C. macellaria* from three different ecoregions in Texas demonstrated significant difference in development rate and size at 21°C and 31°C. Interestingly, while *L. sericata* strains exhibited more variation in development time at 33.5°C, in this study the *C. macellaria* strains exhibited more variation in development time at 20°C.

Such experiments produce information on genetic differentiation in blow flies on a broad scale, but do not provide much information about specific alleles involved in the genetic component of trait variation. Alleles associated with traits of interest can be mapped in an effort to narrow future investigation to phenotypically relevant loci. For mapping, two common techniques are used; genome-wide association studies (GWAS) and quantitative trait locus (QTL) studies. With GWAS, wild derived strains are genotyped and phenotyped to find common alleles associated with differences in phenotypes. A GWAS compares and evaluates inbred lines for a trait of interest against wild or evidentiary samples. One such study has been done on 107 phenotypes of *Arabidopsis thaliana* inbred lines to successfully identify the genetic basis of various phenotypes (Atwell et. al 2010). GWAS focuses on mapping genetic data and finding common alleles, but has both false positives and false negatives due to population structure and the sheer number of tests that must be performed to assess quantitative trait variation. QTL analyses evaluate differences in phenotype typically among crosses of a few natural genotypes, and are conducted at a much lower genomic resolution. QTL research of *D. melanogaster* body size demonstrated greater chromosomal inversion polymorphism of ancestral lines when compared to newer lines, due to selective pressures caused by latitudinal variation (David et al. 2011). This variation validates the importance of the trait and of differences based on the geographical source. To alleviate the false positives in the Atwell et al. 2010 paper, Nemri et al. (2010) incorporated GWAS and QTL to map mildew resistance in F2 populations to focus on candidate SNPs in QTL regions. Utilizing GWAS along with linkage analysis, increases the

mapping resolution of the QTL to the candidate gene level by reducing background, streamlining the gene discovery process. While both QTL and GWAS are beneficial approaches, rearing blow fly lines and inbreeding siblings to narrow down a quantitative trait would be a time intensive endeavor. Another approach to mapping loci associated with a complex trait is to map shifts in allele frequencies that occur during an artificial selection experiment (Burke et al. 2010, Turner et al. 2011).

Artificial Selection

One way to investigate genetic variation in complex traits is an artificial selection experiment. For example, populations of *D. melanogaster* were selected for late life reproduction for over 600 generations (Burke et al. 2010). Selected populations developed approximately 20% faster than the baseline controls and evolved a number of correlated phenotypes including higher longevity. Sequencing revealed high quality SNPs with high allele frequencies among five replicate populations for accelerated development against controls. Selection amplifies phenotypic differences making it possible to measure direct, indirect, or correlated responses (Huey and Kingsolver 1993). As selection experiments have a high level of control over the evolutionary process, it aids our understanding of the physiological mechanisms involved with selection response (Gibbs 1999).

Selection experiments can also allow for a calculation of heritability, through the use of the breeder's equation. Heritability can be determined by a variety of breeding

methods all of which are designed to measures the importance of genetic variance in determining phenotypic variance. Depending on the breeding program and investigation being done, researchers could be analyzing half-sibling relationships, full-sibling relationships, or offspring-parent regression to estimate genetic variance and heritability (Falconer 1981). Previous research established that certain breeds of cattle have a higher heritable resistance to the economically important cattle tick, *Rhipicephalus microplus* ((Canestrini) (Ixodida: Ixodidae); formerly *Boophilus microplus*), than others (Lush 1924). Hewetson (1972) compared this to his work crossing Zebu breeds with others to observe the underlining inheritance of pest resistance. Various breeds were initially susceptible to infestation and then subsequently developed degrees of resistance, with breed and strain shown to have strong effects. Ecological research has also investigated the heritability of female fecundity of the Red deer in the wild and its effect on genetic variation in fitness (*Cervus elaphus* (Linnaeus) (Artiodactyla: Cervidae)) (Kruuk et al. 2000). Life history traits such as fecundity are subject to strong directional selection and have paradoxically lower heritability than morphological traits (Roff and Mosseau 1987). Even with a lower heritability, life history traits can be altered greatly through artificial selection and knowledge of trait heritability is vital to understanding evolution and selection (Burt 1995). Thus a selection experiment can provide a broad overview (heritability) and locus-specific (through mapping experiments tied to selection experiments) information regarding the genetics of a complex trait.

Selection experiments can be used to illustrate how quickly the mean phenotype evolves and responds (Conner and Hartl 2004), as well as heritability of those

phenotypes over generations. Palmer and Dingle (1986) demonstrated how selection can be used to estimate heritability, when the milkweed bug (*Oncopeltus fasciatus* (Dallas) (Hemiptera: Lygaeidae) was selected for mean wing length. After nine generations, a bidirectional response was shown in short and long wing length of male and female milkweed bugs to selection against the non-selected control. Realized heritability was determined from the offspring-parent regression of the cumulative phenotypic response on the cumulative selection differential. The differential was calculated from the difference of the generation's phenotypic mean from the parental phenotypic mean (Falconer 1981). The results for realized heritability were significant, with a heritability of 0.49-0.54 for long wing lengths and 0.23-0.36 for short wing length. The rapid and wide response to selection of *O. fasciatus* confirmed the considerable additive genetic variation (most important for an evolutionary response) of wing length and the complexity of this life history trait. According to Fisher's fundamental theorem on natural selection, the change in rate of mean fitness is proportional to additive genetic variance in fitness for a population at that time (Fisher 1958). Given the ease of rearing *C. macellaria*, with relatively short generation time, selection experiments appear to be a feasible and practical choice for dissecting the genetics of life history traits in this species.

This selection experiment focuses on the change in development rate over more than 20 generations of selection in *C. macellaria*, testing how development time diverges within and among three wild type populations. From the selected adult data, offspring-parent regression estimates the heritability of fast and slow development

alleles. The initial generation consisted of adult flies (G0) collected from three different locations in Central Texas that served as biological replicate populations and as a genetic baseline. The flies were bred within their respective experimental population and their immature progeny reared at 25°C. Once the first generation emerged, adults from each of the three experimental populations were selected and separated into fast and slow developing flies, to produce six selected lines. Development time was measured as hours from eggs collected to adult eclosion. These selected lines and samples from future generations can be screened against each other for later genetic analysis. Genetic markers could ultimately identify fast or slow developing indicators of wild type *C. macellaria*, aiding in tightening age estimates on blow flies.

The selected lines of *C. macellaria* colonies were produced then split and reared at three temperatures 20°C, 25°C, and 30°C to evaluate the impact of selection on plasticity and life history trait correlations. The resulting reaction norm demonstrates the plasticity of development speeds at different temperatures of the selected lines. This portion of the study demonstrates the differences in thermal responses between alleles that were selected in fast or slow selection regimes. Pupal mass and survival data were also collected at the three temperatures and scored to find correlations among factors: sex, development rate, selection speed, experimental populations, and temperature.

These studies, in addition to providing evolutionary ecology information for understudied ephemeral resources, on the development of *C. macellaria* would expand and continue to facilitate forensic entomology, especially as it relates to error in the

field. A mandate was set by *Daubert v. Merrell Dow Pharmaceuticals, Inc.* (1993) that certain criteria need to be met prior to evidence being allowed into court: the evidence must be testable, have a known error rate, data used must be peer reviewed, and be accepted by the specific scientific community (Tomberlin et al. 2011b). The U.S. Supreme Court stated the criterion was not a solid checklist so that future rulings would be more malleable in deciding the scientific validity. The latter decision has caused problems for judges unfamiliar with scientific techniques, who yet must determine if evidence is permissible (NAS/NRC 2009). Over time, a number of established practices of forensic sciences were unable to meet the criteria, and scientists called out for reform to improve the model, striving to meet the "gold standard" that is DNA analysis (Saks and Koehler 2005). In a 2009 report the National Research Council/National Academy of Science criticized the forensic sciences, detailing the need for further training, better and consistent lab practices, and quality control (NAS/NRC 2009). In forensic entomology, an evolutionary ecology approach to research in the field, considering genetic and environmental factors that are not currently accounted for as sources of error, has been proposed as a means of improving the discipline (Tomberlin et al. 2011a). Utilizing a rigorous selection process and through consideration of evolutionary ecology of thermal biology, the development of these selected strains will advance our understanding on the basic biology of *C. macellaria* phenotypes. The hope is that this improved understanding will be informative to a number of fields, including ecology, evolutionary biology, and forensic entomology.

CHAPTER II

SELECTION RESPONSE OF DEVELOPMENT TIME

Introduction

Ever since the domestication of animals and agriculture, humans have benefitted from utilizing artificial selection. We select traits for social and public interest, including breeds of dogs designed for appearance (Stearns and Hoekstra 2000) or performance of specific tasks (Ostrander and Kruglyak 2000), cattle that produce more meat or milk (Carabaño et al. 1990), and corn that bears more oil (Dudley and Lambert 2004). With natural selection such traits are rarely, or never, found in the wild, but consistent artificial selection can drive biological evolution in a chosen direction and expose the limits of natural genetic variation. This variation is an indication of the evolutionary potential for a given trait of interest. While animal breeders want to exploit an economically important trait, evolutionary biologists seek to understand the path that molded that trait (Hill and Kirkpatrick 2010). The two approach the problem from different perspectives; yet both study the same genetic machinations that drive evolution of phenotypes. Charles Darwin saw the connection between animal breeding and evolution and discussed how artificial selection is analogous to natural selection in his book, *On the Origin of Species* (Darwin 1859). “Survival of the fittest” has less to do with who is the fastest or the smartest, but who adapts the best for the species to survive.

This ties in well with the concept of evolutionary fitness, which is intimately connected to organismal phenotypes and dependent on the environment and genetics of an organism (Burt 1995, Kingsolver and Huey 2008). Thus, a trait may evolve in the wild due to natural selection on a fitness related phenotype that enhances evolutionary fitness (the ability to produce more offspring than others) in a particular environment. In an artificial selection experiment the breeder manipulates fitness to achieve a desired trait.

Examples of both natural and artificial selection are common in biology literature. A classic example of natural selection occurred with the moth, *Biston betularia*, during the Industrial Revolution. Darker moth populations thrived in urban environments due to the ubiquitous amount of soot in their habitat, while lighter moth populations survived, better protected from predation, in natural environments. Moths that adapted to their environment selected for a specific camouflage to avoid predation by successfully blending into their environment (Cook and Saccheri 2013). In natural systems fitness determines evolution of a phenotype instead of a single minded trait breeder. Natural systems consequently evolve differently, as fitness can often be impacted by multiple traits at once, adjusting back and forth in an ever-fluctuating environment.

One important concept to consider when evaluating a selection experiment is that of standing genetic variation, that is, the genetic variation in the population that is available for selection. As an example, Thoroughbred horses have been artificially selected for speed and stamina for the past 300 years, making them superior competitive racehorses. The starting variation in the populations that founded the breed was derived

from wild horses where being fast could protect an animal from predation. However, the ability for a trait to evolve (in the wild or in an artificial experiment) depends on the genetic variation in the population. There has been little improvement in winning times of Thoroughbreds in the past 50 years, as the fastest record at the Kentucky Derby has been held by Secretariat since 1973 (Hill and Bünger 2004). One hypothesis explaining this observation is that the genetic variation of the base populations originating from Arab, Barb, and Turk stallions and native British mares has been exhausted and that all the available variation has been fixed in the past 50 years (Cunningham et al. 2001, Hill et al. 2002, Bower et al. 2011). Alternatively, selectively bred homozygotes for speed could be a limitation if heterozygosity results in greater speed. As an example, one study demonstrated that SNP variation at a locus influenced skeletal muscle development and racing speed, with homozygotes having an aptitude for short or long distances, while heterozygotes have an aptitude for middle distance races (Hill et al. 2012).

Artificial selection can direct variation of traits in a controlled manner, but the selection process does not necessarily reflect what is found in a natural population (Harshman and Hoffmann 2000). If environmental fluctuations are removed from the equation, this could limit the genetic potential that would allow for a faster speed phenotype and its heritability in future generations (Parsons 1983, DeWitt 1998). As a trait evolves within a wild population, gene by environment interactions influences this genetic variation, and this is an integral factor for expressing a phenotype that is important for understanding the evolutionary dynamics of wild organisms (Clare and

Luckinbill 1985, Luckinbill and Clare 1985). Thus selection on a trait is not necessarily expected to produce the same results in different environments.

The focus of this thesis is a selection experiment on the development rate of *C. macellaria*. I started with three experimental populations of the secondary screwworm to act as biological replicates and as a baseline to check against the selection regime on the phenotype of development. By controlling the environment in the lab, we limit those variables to focus on the genetics that influence a phenotype of interest. This allows us to observe the additive genetic variation of a trait and its response to selection. As the selection experiment progressed, the heritability of fast and slow development demonstrated the extent of variation from those initial populations (Relyea 2005) and could be calculated by the breeder's equation (Falconer and MacKay 1996). From this we can further understand the basic biology of an organism and see how it progresses over generations, offering a better understanding of the evolutionary biology of development. The life history trait of development is an important aspect of fitness, as seen in previous *C. macellaria* research. The initial colonization and interaction of *C. macellaria* among other colonizers has effects on fitness as well as abundance (Brundage 2011). Life history trade-offs are made during the larval stages on the ephemeral resources, and management of nutrients determine the developmental response to starvation (Mohr 2012). Interactions of *C. macellaria* and *C. rufifacies* larva on a resource influence the development of both (Flores 2013). By researching and investigating the life history trait of development of *C. macellaria*, my goal is to find the extent of natural variation for development, determine how wild flies compare to

selected lines, and calculate the heritability of the selected lines for fast and slow development. Such information will provide a useful genetic perspective that future work on life history biologists can apply to interpretations of their work. With the *C. macellaria* research data and further genetic application, a greater understanding of the genetics of development rate could be utilized in forensic entomology to help account for uncertainty in estimates of immature insect ages that can be informative of PMI.

Materials and Methods

Collection Sites

The founding populations of *Cochliomyia macellaria* (G0) were collected from three central Texas sites in June and July 2011. Adults were collected by a sweep net, while larvae and eggs were hand collected from carrion or liver bait. From June 16-18th, 2011 *C. macellaria* were collected from three different sites within Longview, TX by Charity Owings, M.S. using bovine liver and one raccoon (found roadkill) as bait. From July 6-8th, 2011, *C. macellaria* were collected from Snook and College Station, TX by Dr. Christine Picard, using pig (*Sus scrofa*) carcasses as bait. The resulting blow flies were bred within their experimental populations to produce the first selected generation, G01 in the Texas A&M University Forensic Laboratory for Investigative Entomological Sciences (F.L.I.E.S) facility.

Longview (32°30′33″N 94°45′14″W) is in northeast Texas in the humid subtropical East Texas Pineywoods ecoregion. College Station (30°36′05″N 96°18′52″W) and Snook (30°29′25″N 96°28′11″W) are in central Texas in the subtropical and temperate Texas Post Oak Savannah ecoregion. In the months G0 was collected, the average temperature ranged from 27-29°C; below is a table of average weather data.

Table 2.1. Average temperature and precipitation data of the three collection sites and the month of *C. macellaria* collection.

Texas Location	Collection Month / Year	Average High	Average Low	Mean	Average Precipitation	Average Warmest Month	Average Coolest Month
College Station	July 2011	35°C	24°C	29°C	54.4mm	August	January
Longview	June 2011	32°C	21°C	27°C	112.8mm	July	January
Snook	July 2011	33°C	23°C	28°C	113mm	August	January
Data collected from www.weather.com							

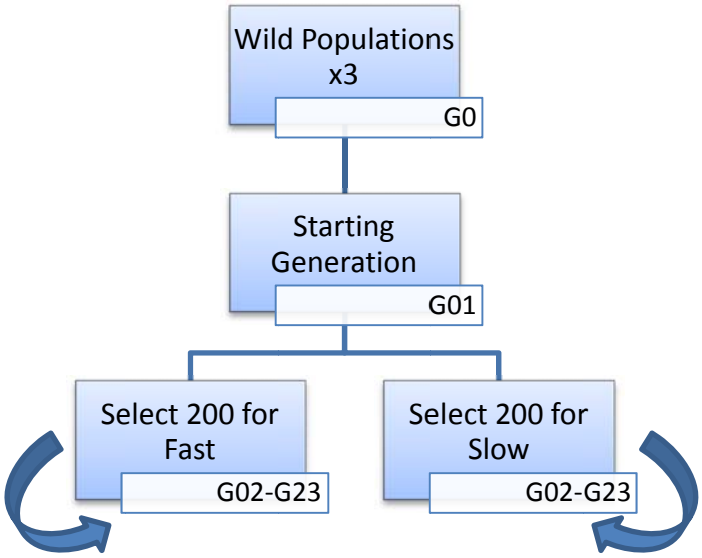


Figure 2.1. Experimental design for selection. Three Texas wild populations were collected and reared in the lab to produce the G01. From G01, 100 males and 100 females were selected to produce the G02 lines. The 200 fastest produce the G02 fast lines, and the 200 slowest produce the G02 slow lines. Selection repeats for the duration of the experiment.

Experiment 1: Selecting for Development rate of C. macellaria

All experimental lab work was done in the Texas A&M University Forensic Laboratory for Investigative Entomological Sciences (F.L.I.E.S) facility. The above flow chart generalizes the experimental design for selection (Figure 2.1). The G0 adult flies were maintained in BioQuip cages (BioQuip 1452 Bug Dorm, 11.75" cube) at approximately 27°C and provided with water and sugar *ad libitum*. The three Texas experimental populations were each given approximately 5mL beef liver blood as a protein source for approximately 3-5 days. For egg collection, a 3 ounce plastic cup containing 1mL of distilled water, 2 Kim-wipes and approximately 20g of bovine liver

were put into each cage at 7am of each day to promote oviposition. Liver cups were checked at 11am, 3pm, or 7pm for egg collection. Approximately 1,200 eggs were needed for each generation from each experimental population. Blow fly eggs were collected and arbitrarily picked and put in six glass Mason jars (1-quart size), with an estimated 200 eggs in each. The six Mason jars per treatment per experimental population were half-filled with vermiculite substrate (Premium Grade, Sun Gro Horticulture F1153) with a small piece of paper towel on top to hold approximately 50g beef liver. The 200 eggs were laid on the liver, then covered with a Kim-wipe to retain moisture, and reared at 25°C with 50% RH on a 14:10 L:D cycle in a controlled environment incubator (136LLVL Percival, Percival Scientific Inc.). Mason jars were covered with a Wypall paper cloth (Kimberly-Clark Global Sales, LLC) and sealed with a Mason jar lid ring. Temperature was monitored using HOBO data logger (ONSET HOBO U12-006) and actively checked using an incubator thermometer (VWR # 89095-800) when the incubator was opened. Date, collection time, and experimental population for each jar were noted. Development time began with the onset of egg collection.

With the onset of pupation, pupae were counted and put into cylindrical mosquito breeder cages (BioQuip 1425, 8-3/8" x 4-7/8") for eventual eclosion. Mosquito breeder cages were checked four times daily: 7am, 11am, 3pm, and 7pm. When adults emerged, the data logged for each collection at each time point for each selection line and rearing temperature was eclosion time, number of males, and number of females. To separate the adults from the remaining pupae, the cultures were chilled at 4°C for approximately 5 minutes or -20°C for approximately 2 minutes. This cooling time was used throughout

the experiment to make the adults more manageable for counting. This data collection process was carried out four times a day over the course of the eclosion, which ranged from two–seven days. The first 100 males and 100 females were selected to make fast developing lines. The final 100 males and 100 females were used to start the slow developing lines.

Adult *C. macellaria* were established and maintained as six strains (six cages) for each selection regime and experimental population, and once the requisite 100 flies of each sex were acquired for each cage, blood meals were provided to aid egg maturation. In subsequent generations, blow fly eggs were collected as before, but only fast were selected from the fast lines and slow from the slow selected lines. This selection regime was repeated for 18 months resulting in 29 generations (G29) of fast lines and 23 generations (G23) of slow lines for College Station and Snook experimental populations, while Longview achieved G30 for fast and G24 slow lines. Every generation, after progeny hatched, the previous adult generation was frozen and stored at -80°C for later genetic analysis.

The rearing substrate was changed to sand (Quikrete 50lbs Play Sand, Item# 10392, Model# 111351) at generation seven of the Longview strains and generation six of College Station and Snook strains. This was predominantly done to save time during separation of pupae.

Statistical Analyses

Statistical analyses were done with JMP 10.0 on the mean development time, in hours, of each line at each generation. By using the average development time, from egg collection to adult emergence in hours, each generation from each line constituted a single data point. While 1,200 flies was the egg collection goal, numbers varied over the generations for the 18 months of selection. To prevent biases in statistical analysis due to generations with varying number of adults and avoid overconfidence in results due to large samples, a single average per generation per line was used for the ANOVA. JMP performs Type III ANOVA.

ANOVA was done to assess the effects of selection, experimental population, generation, and sex (as well as their interactions) on immature development time. Student's t-tests and Tukey's HSD (Honestly Significant Difference) tests were used to determine significant mean differences within the categories ($p < 0.05$). Eta squared (η^2) was calculated in Excel to observe and compare effect size (%) of individual factors on the development ANOVA models. The η^2 was determined by the factor of interest sum of squares/total sum of squares, to offer a simple overview of each factor's relative contribution to the ANOVA model (Sechrest and Yeaton 1982). In this chapter, I ran three series of model assessments: development between the sexes, substrate effects on development, and overall development. Model assessments were organized from highest to lowest AICc (Akaike information criterion) to show improvement of model quality; the R^2 shows how well the model explains the data.

MANOVA analysis was performed with a random subset of data from each generation per selection group per line in order to eliminate issues of imbalance in the data (different numbers of flies per generation). The size of the subsets was determined by the lowest number of blow flies in a generation of the selected lines, which was 229 blow flies for the Longview fast G16. Rounding down, data for 200 blow flies (100 males and 100 females) from each line for each of the 23 generations was randomly picked by JMP 10.0, and then nested within each generation to accommodate the MANOVA dataset requirements for analysis. This added up to 27,600 blow flies, approximately 17.2% of the complete raw data. The MANOVA analysis was done to account for temporal autocorrelation in the experiment and to determine if autocorrelation had an impact on the results seen in the ANOVA.

Calculating Heritability

During the selection process, the eclosion time of each blow fly was logged, including those selected for breeding. From this, the mean time of development at each generation and the mean development time of those bred were calculated for every selected line. With this, heritability was determined using the breeder's equation, $R = h^2S$, a fundamental equation describing phenotypic evolution, in which R (response to selection) equals the h^2 (heritability) times S (selection differential). The phenotype being selected for was the development speed (in hours), which was determined by the average time from when the *C. macellaria* eggs were collected until it emerges as an adult. The S represents the difference of the mean development time of all the blow flies

from each generation and the mean development time of breeders selected to produce the next generation. The response represents the mean change in development time between the offspring's generation and the previous generation. Over time, selection shifts the average development time, decreasing the average for fast and increasing the average for slow. By using the examples from *Introduction to Quantitative Genetics* by Falconer and Mackay, 4th edition (1996), heritability over multiple generations was determined by the slope of selection differential (x) by response (y). Response (R) was simply the mean development time of a line over generations. The selection differential was determined by the accumulated selection difference (S) added together over generations. For an example, please see APPENDIX A: CALCULATING HERITABILITY.

Results

Selection: Divergence of Fast and Slow

Selection appeared to alter development times for all experimental populations studied. When comparing the experimental populations at the 23rd generation, the aggregate difference between fast and slow development was 121.2 hours, or approximately 5 days (Figure 2.2), with an asymmetric selection response wherein faster developmental shifts were not achieved at the same rate as slow development. Table 2.2 lists the mean development time and its respective variance, both calculated through JMP 10.0 on the raw data. The variance in development time decreased with fast selection while increasing with slow selection, for both College Station and Snook experimental populations. For comparison, a Fisher's Exact test demonstrated a significant difference in the variance between fast lines and slow lines for all three experimental populations at G23.

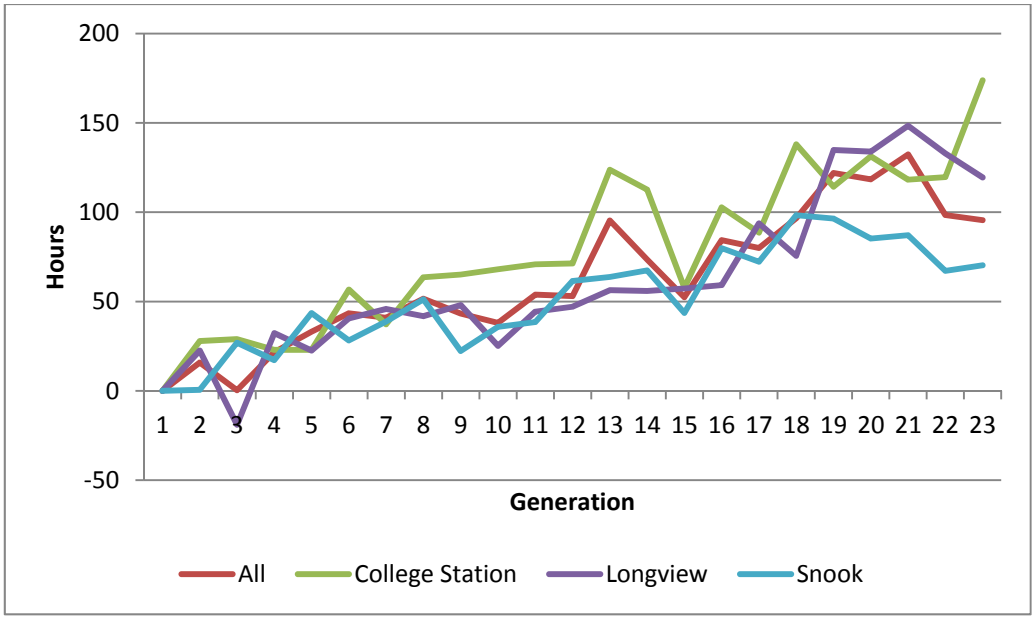


Figure 2.2. Difference in average development time between selected lines. Fast and slow selected line comparison, in hours, for 23 generations. College Station, Longview, and Snook points were the difference in means of fast and slow at the listed generation. All is the average difference among the three experimental populations.

Table 2.2. Mean development time and variances (Var) of selected lines. Average development time and its difference in hours, as well as variance in development time of experimental populations and the combined averages, from G01 to G23.

	G01 Mean	G23 Slow Mean	G23 Fast Mean	Δ G23		G01 Variance	Var G23 Slow	Var G23 Fast
College Station	259.3	418.5	244.6	173.9		219.3	1202.2	151.5
Longview	284.3	366.8	247.3	119.5		277.1	531.6	324.9
Snook	273.1	307.2	236.9	70.3		257.9	685.0	64.3
All	270.4	364.1	242.9	121.2		344.2	2212.0	234.4

The *cinnabar* phenotype

A *cinnabar* mutation was observed in the Longview slow developing line from G03-G15 (Figure 2.3), with the mutation's phenotype frequency ranging from .63% to 10.27% of flies per generation. As the selection process progressed, the mutation was replaced by wild type eye color. This suggests that the mutation was linked to a target of selection, but that *cinnabar* itself was not under selection.

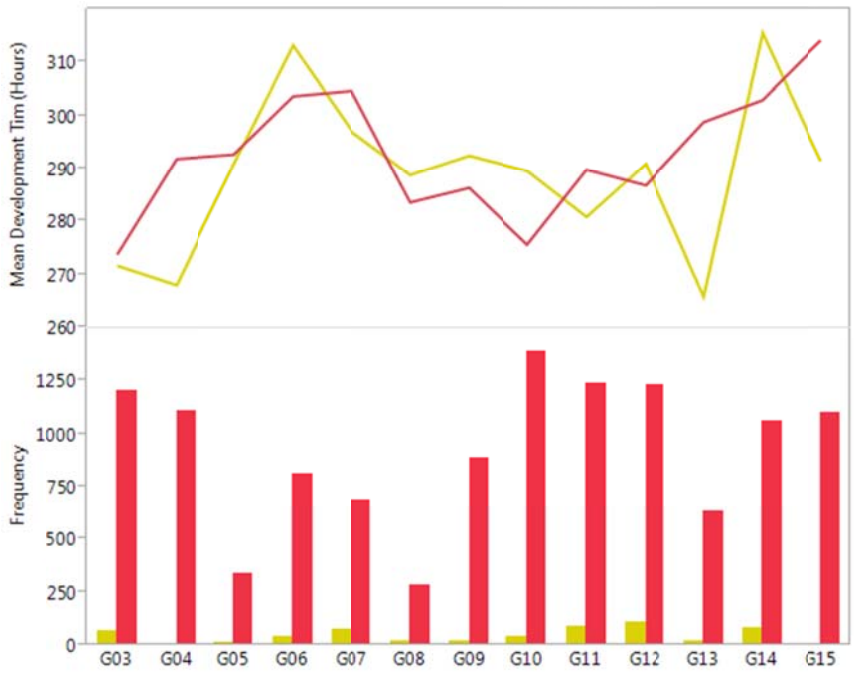


Figure 2.3. Mean development time and frequency of cinnabar (*cn*) mutant. Longview slow selected line showing the difference in development rate in hours (y-axis, top row) and frequency of eye color (y-axis, bottom row) over generations (x-axis). Wild type eyes shown in red and *cn* mutants in yellow.

Model Assessments

A number of ANOVA statistical models were run to analyze the overall developmental data presented in this study. Area model selection process was run using JMP 10.0 to determine the most likely models that explain development time variation. The R^2 was included to explain how informative the factor(s) included in the model were in explaining the predicted variable. The AICc measure reflects the likelihood that a model explains the data better than other models, with more likely models resulting in lower AICc scores. AICc scores within two units of one another were considered indistinguishable. The factors tested in the models were generation, genetics from selection, and genetics from experimental population; additional factors of substrate and sex were included for the respective datasets. The use of the term "Population" in the following model assessments and ANOVAs refers to experimental populations in this study, and is not intended to be extrapolated out to the entire population from which the experimental population is derived. A total of three different datasets were utilized, to test three aspects acting on development in the selection experiment: substrate effect, development rate differences between the sexes, and overall development rate.

Model Assessment: Between the Sexes

The development time between the sexes were assessed by the factors of genetics (both by the selection regime, which reflects within-group genetic variation and by experimental population, which reflects between group genetic variation), generation, and sex. Several mixed model ANOVAs, with sex as a fixed effect, were evaluated. In the model with the lowest AICc, selection demonstrated the largest effect size of the single factors. When sex was in included within a model, it was not a significant source of variation and exhibited the weakest η^2 of 0.2%. While males developed, on average, up to 5 hours faster than females, sex was disregarded in later model assessments due to the relatively small impact on developmental variation and its insignificance in models that included it as a parameter.

Model Assessment: Substrate on development

Table 2.3. Model assessment of development from rearing substrate. Models assessed utilized a single dataset of mean development rate per generation of the selected lines, for the first 14 generations, excluding the substrate factor.

Model	Development Time (hours) =	R^2 (%)	AICc	p- value
1	Generation + Population	14.5	830.2	0.7686
2	Generation	9.7	828.6	0.5774
3	Population	4.8	804.4	0.1357
4	Selection + Generation	62.7	757.5	<0.0001 *
5	Selection + Generation + Population	67.5	752.3	<0.0001 *
6	Selection + Population + Generation + (Sel. x Pop.)	70.5	750.8	<0.0001 *
7	Selection	53.0	743.0	<0.0001 *
8	Selection + Population	57.8	738.4	<0.0001 *
9	Selection + Population + (Selection x Population)	60.8	736.8	<0.0001 *

To determine the substrate's impact on development, models were first assessed using factors other than substrate to explain development time (Table 2.3). Residuals were taken from models 5 and 6, as these models explain the greatest proportion of developmental variation, while still including generation. The residuals from two of these models were run against substrate to reveal the probability of changes on development caused by substrate (Table 2.4) and plotted in Figure 2.4. Model 9, which did not include generation, was not assessed further as substrate was confounded with generation (different substrates used in different generations of the selection experiment), which would make evaluation of residuals from that model difficult to interpret. The ANOVAs run on residuals produced from models (5 and 6) that removed the effects of selection, generation, and experimental population, demonstrated that the change in substrate likely did not have a significant impact on development in the study (Table 2.4).

Table 2.4. ANOVAs for residuals to determine if substrate type was associated with developmental differences. Three ANOVAs output of development time and the effects of generation, selection, its interaction, and experimental population.

Phenotype	Model	Source	Df	Sum of Squares	Mean Square	F ratio	Prob. > F
Residuals [Model 5] $R^2 = 0.2$	ANOVA	Model	1	65.274	65.274	0.2450	0.6219
		Error	82	21843.107	266.379		
		Total	83	21908.380			
	Effect Test	Substrate	1	65.274		0.2450	0.6219
Residuals [Model 6] $R^2 = 0.3$	ANOVA	Model	1	65.274	65.274	0.2705	0.6044
		Error	82	19788.514	241.323		
		Total	83	19853.787			
	Effect Test	Substrate	1	65.274		0.2705	0.6044

Figure 2.4. Plotted residuals (y-axis) over generations of time (x-axis).
Selection + Population + Generation (blue, p = 0.6219)
Selection + Population + Generation + (Selection x Population) (red, p = 0.6044)

Model Assessment: Overall Development

All mean data was assessed using ANOVA to determine the most likely model to explain development time variation in the experiment and to determine the variables that contributed most to development time evolution in the study. The individual factors of selection and generation, the interaction of selection and generation, and experimental population offer the best model fit for the overall developmental data (Table 2.5, model 13) of *C. macellaria* under the selection regime. Within that model, selection demonstrated the highest effect size of the single factors.

Table 2.5. Model assessment of overall development. Models assessed utilized a single dataset of mean development rate per generation of the selected lines, for the first 23 generations.

Model	Development Time (hours) =	R^2 (%)	AICc
1	Generation + Population + (Population x Generation)	17.4	1684.8
2	Selection + Generation + Population + (Selection x Generation) + (Population x Selection) + (Population x Selection x Generation)	95.1	1630.7
3	Population + Generation	12.5	1469.0
4	Generation	8.9	1468.5
5	Population	3.5	1426.1
6	Selection + Generation	67.6	1328.9
7	Selection + Generation + Population	71.2	1319.0
8	Selection + Generation + Population + (Population x Selection)	72.9	1316.6
9	Selection	58.6	1307.3
10	Selection + Population	62.2	1299.2
11	Selection + Population + (Selection x Population)	63.9	1297.0
12	Selection + Generation + (Selection x Generation)	86.3	1292.8
13	Selection + Generation + (Selection x Generation) + Population	89.8	1260.9

The ANOVA model for development time over the first 23 generations shows a significant difference between fast and slow selection regimes ($p < 0.0001$, Table 2.6) across the three experimental populations. The fast selected lines averaged 1.5 days faster than G01 and slower took an average 3.5 days longer. The Tukey's HSD test listed significant differences between all three experimental populations.

Table 2.6. ANOVA for overall development. Model 13 in table 2.5. ANOVA output of development time and the effects of generation, selection, its interaction, and experimental population.

Phenotype	**Model**	**Source**	**Df**	**Sum of Squares**	**Mean Square**	**F ratio**	**Prob. > F**	**η^2 %**
	ANOVA	Model	47	218104.70	4640.53	16.94	<0.0001 *	89.8%
		Error	90	24652.45	273.92			10.2%
Development		Total	137	242757.14				
	Effect Tests	Generation x Selection	22	45361.639		7.528	<0.0001 *	18.7%
R^2=0.898		Generation	22	21825.925		3.622	<0.0001 *	8.9%
AICc=1260.9		Selection	1	142283.86		519.443	<0.0001 *	58.6%
		Population	2	8633.275		15.759	<0.0001 *	3.6%

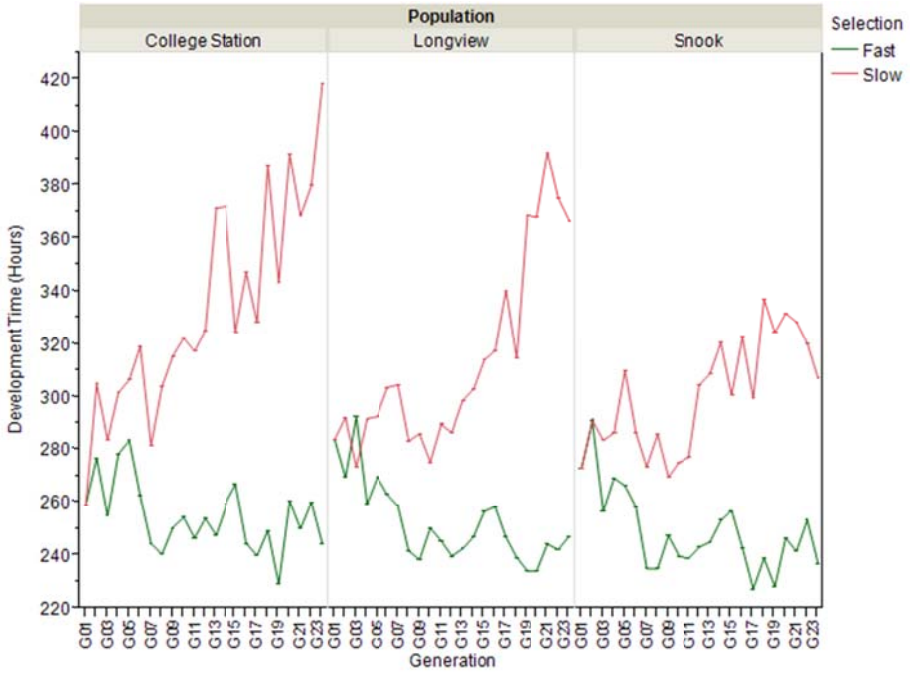


Figure 2.5. Mean divergence of development time of selected lines. The three experimental populations are in columns with mean development time in hours (y-axis) for 23 generations (x-axis); fast in green and slow in red. The first generation, G01, averaged among the three experimental populations presented a mean development time of 270.4 hours.

Heritability of Development Time

Heritability of development time was calculated using the breeder's equation. The slopes representing heritability (Figure 2.6) were produced from regression of cumulative difference in hours of development time between the average of the experimental population and the average of the selected breeding adults over 23 generations (x-axis) against the response of selection, i.e. the mean development time, at each generation. Based on the longer sigma selection, slow developing lines (red) exhibited greater additive genetic variation, and therefore heritability, in development time than fast developing lines (green). Faster lines changed less, such that the slopes of the green lines and confidence intervals were smaller and heritability less over the 23 generations. Table 2.7 below, lists the progressive change in heritability at different generation milestones within this study. The G06 was the last generation immatures were reared in vermiculite, G15 showed a plateau in Snook slow selection, and G23 was the final generation analyzed in this experiment. The shift illustrates effects on heritability in response to selection over generations.

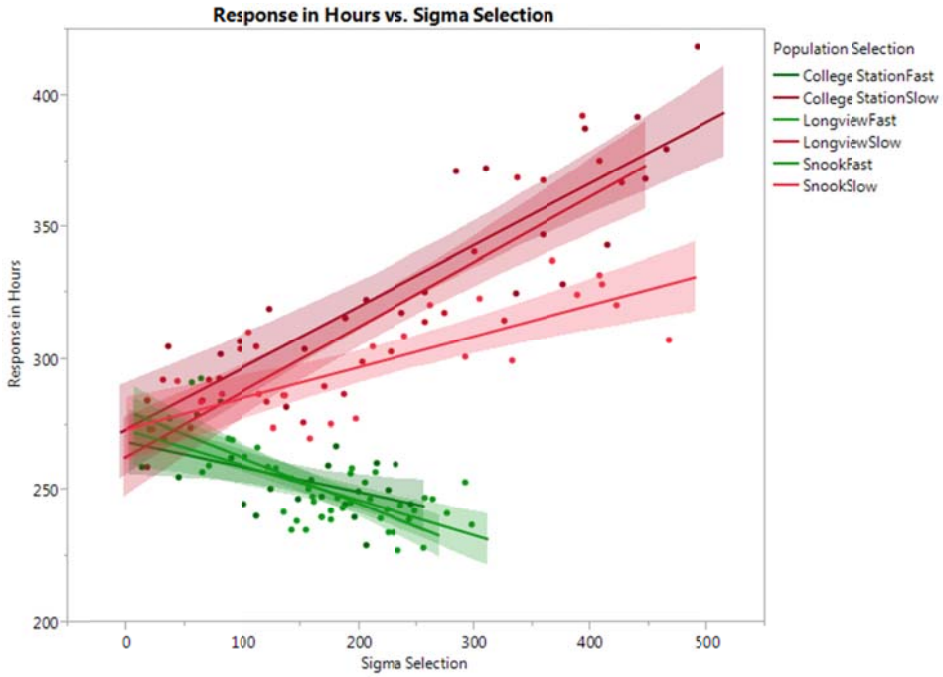


Figure 2.6. Sum of selection response over 23 generations for fast (green) and slow (red) development for three experimental populations. Sigma selection (x-axis) was the cumulative difference of parental and generational means versus response as the regression of selection for mean development time in hours (y-axis). For heritability calculation example, see APPENDIX A: CALCULATING HERITABILITY.

Table 2.7. Heritability (h^2) for slow and fast development. Columns list generation range from G01 to G06, G15, and G23 of the six selected lines. Generations were determined by milestones in the experiment. G06 was the last generation immatures were reared in vermiculite, G15 showed a plateau in Snook slow selection, and G23 was the final generation analyzed in this experiment.

Population	**Fast h^2**			**Slow h^2**		
	G01-G06	G01-G15	G01-G23	G01-G06	G01-G15	G01-G23
College Station	0.156	0.096	0.096	0.428	0.234	0.234
Longview	0.184	0.236	0.176	0.188	0.073	0.247
Snook	0.146	0.188	0.134	0.213	0.097	0.118

MANOVA Statistical Analyses

Multivariate analysis of variance (MANOVA) was performed on the development time for the first 23 generations using 200 randomly selected blow flies (100 male and 100 female) per selected line, per generation. MANOVA was useful to examine multiple, dependent variables between and within generations. The analysis could also account for temporal autocorrelations produced because offspring were not genetically independent from their parents. The blow flies were randomly picked by JMP 10.0. From each generation, 200 were chosen, rounding down from the 229 blow flies present in the G16 Longview fast selected lines. The MANOVA was used to analyze the development time nested within each generation, as experimental population, selection, and their interaction affect it. The MANOVA indicated significant variation among the lines in their overall development time over the generations (Wilk's $\lambda = 0.0034$, $p < 0.0001$). As presented in earlier ANOVA models, relative effect sizes among model parameters were highest for selection and lowest for the experimental populations, based on F-value sizes in these analyses. Due to the limited relative impact of sex and substrate, these factors were not included in the MANOVA.

Table 2.8. MANOVA multivariate and univariate output from JMP 10.0. Generation nested data for the six selected lines, includes the subjects of generation (Gen), selection (Sel), and population (Pop).

Between Subjects	Test	Value	Exact F	NumDF	DenDF	Prob>F
All between	F Test	13.352	3188.480	5	1194	<0.0001*
Intercept	F Test	956.483	1142040.3	1	1194	<0.0001*
Population	F Test	0.738	440.309	2	1194	<0.0001*
Selection	F Test	12.251	14627.344	1	1194	<0.0001*
Pop x Sel	F Test	0.364	217.218	2	1194	<0.0001*
Within Subjects	Test	Value	Approx. F	NumDF	DenDF	Prob>F
All Within Interactions	Wilks' Lambda	0.0002	236.333	110	5751.3	<0.0001*
Generation x Pop	Wilks' Lambda	0.0234	295.510	44	2346	<0.0001*
Gen x Pop x Sel	Wilks' Lambda	0.0676	151.814	44	2346	<0.0001*
Within Subjects	Test	Value	Exact F	NumDF	DenDF	Prob>F
Generation	F Test	21.936	1169.569	22	1173	<0.0001*
Generation x Sel	F Test	26.187	1396.235	22	1173	<0.0001*

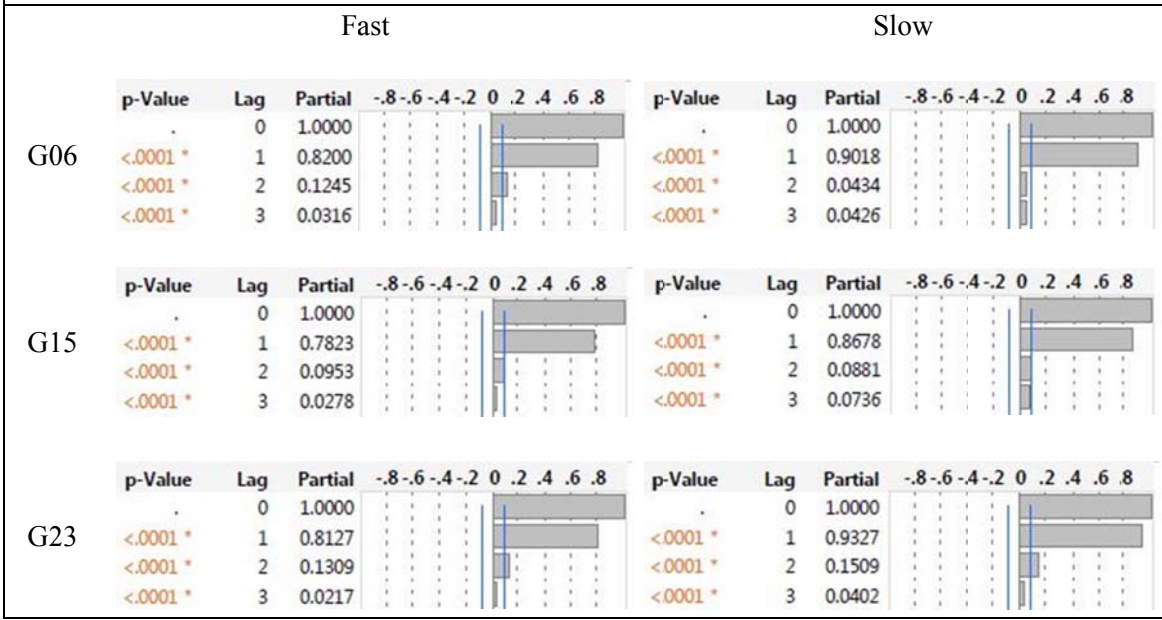
An additional MANOVA was run using the same dataset separating the analyses by fast and slow selection (Table 2.9). From the additional analysis, significant differences could be observed among the experimental populations within the fast selected lines and slow selected lines. When the analysis was repeated, focusing on G01 through G15, approximately when the Snook slow line plateaued, the MANOVA displayed the same result. There was a significant difference in development within the fast lines and the slow lines for the experimental populations between generations ($p < 0.0001$). The model was repeated for the first six generations, G01 through G06, as this was when fast and slow lines diverged enough in development patterns to be grouped separately for selection. Similar results were observed for the first six generations as for the first fifteen generations. Overall, there was a significant difference in development through all generations in the selected lines.

Table 2.9. Additional MANOVAs to observe development nested within the generations. Analysis was done by selection to observe differences within fast and slow developers among the different populations.

Y	=	Factor (s)	Test	Fast	Slow
G01 to G06	=	Population	Wilk's λ	$p < 0.0001$ *	$p < 0.0001$ *
G01 to G15	=	Population	Wilk's λ	$p < 0.0001$ *	$p < 0.0001$ *
G01 to G23	=	Population	Wilk's λ	$p < 0.0001$ *	$p < 0.0001$ *

The MANOVA models described above were analyzed via a time series model (Figure 2.7) to observe the effect of temporal autocorrelations on development time. The data demonstrated a significant difference ($p < 0.0001$) in development after a 1 generation lag, indicating that the genetic component of a parental generation influences the variation in immediate offspring development. The time series output also showed a weak grandparental effect (lag 2) on development time evolution in some but not all cases. The impact of grandparental genotype was considerably smaller than the parental effect and not always statistically significant.

Figure 2.7. Time series output of G06, G15, and G23 using MANOVA dataset. Partial correlation presented similar results for all 23 generations and experimental populations of the selected lines.



Discussion

Over the course of the 18-month selection experiment, 160,234 flies were reared, sexed, counted and phenotyped. The goal was to rear approximately 1,200 blow flies of each line for every generation. As noted in the red flour beetle (*Tribolium castaneum* (Herbst)(Coleoptera: Tenebrionidae)) experiment, population size demonstrated greater genetic and phenotypic drift in smaller populations (Rich et al. 1979), and the effects of genetic drift impact the variance around the phenotypic trajectory in a selection experiment by increasing variance around the trajectory as population size decreases. The selection response in this experiment was not smooth; suggesting that either drift was contributing to variation in the selection response or that there was an undefined source of error in the experiment that impacted the accuracy of measurement of development time. Drift may be a possible explanation, as 1,200 eggs reflects a minimum of approximately six founding adults per generation as females lay hundreds of eggs. While this was an option, as generations went on, egg collection required more time points to achieve the desired numbers, necessitating more females over time to produce the next generation. In the initial generations, egg collections were done at two time points over one day to accumulate the needed eggs for succeeding generation. Closer to G20, faster lines would take require collection at three time points over two days and at least four time points over three days for slower lines. This could result in greater phenotype measurement error. The goal of the selection process was to influence development, but likely had a pleiotropic response on egg production that was not

investigated in this study. For adults, eclosion time was logged at either 7am, 11am, 3pm, and 7pm. Due to the large number of continuously eclosing adults, blow flies were consistently grouped closest to their time point, while trying to accurately log the development of multiple lines emerging simultaneously. Obviously, this process could lead to errors in assigning individuals as "fast" or "slow" developers within the lines and could impact the genetic response across generations. Also, the selected adults were housed in a colony room, which, while controlled, had occasional fluctuations in temperature that could have influenced the adults and their subsequent offspring.

Relative Effect Sizes of Factors on Development

While it was important to observe which factors were significant variables associated with development time selection, it is also important to evaluate the sizes of effects noted in the experiment. Selection had the strongest effect size (as measured by η^2) on development time supporting the biological importance of genetics on development within these lines. The second highest effect was generation, suggesting the potential role of genetic drift in shifts of the phenotype or error if experiments suffered from a systematic problem in measurement of development time. At the 23rd generation, a five-day difference was present between fast and slow development among the three experimental populations. The experimental populations significantly impacted developmental variation within the *C. macellaria* species, yet it had little effect on overall development times compared to the selection genetics. From this study, we must ask if the development time was distributed within or between the experimental

populations. The data demonstrated each experimental population offered substantial genetic diversity within each group. The significant difference among the experimental populations demonstrated minor between group genetic variation, observed in the increased variance of the fast Longview line (Table 2.2) and developmental plateau of the slow Snook line (Figure 2.5). The remaining factors considered here, experimental population, eye color, and substrate had a negligible effect on the development of *C. macellaria*.

Sex

Sex differences in development time appeared to be negligible in *C. macellaria*. Forensic entomologists are limited on samples and have a wide array of variables to contend with in real time. Thus, directing their efforts towards sex effects on development would not be useful within this species, based on this study, until other more important factors are considered.

The *cinnabar* mutation

The *cinnabar* mutants persisted up to the 15th generation of the Longview slow line. The G0 adults had *cn* mutants present, but they were only measured between G03-G15. The phenotypic frequency ranged from .63% to 10.27% of flies for each generation and had a negligible effect on development. Initially, I believed there could be both a genetic and a physiological linkage between the *cn* mutation and alleles related to slow development. As the trait was eventually bred out of the selection process, it appears more likely that there was merely a linkage association between *cn* mutation and slow

development alleles that was eliminated through recombination over time. Alternatively, drift led to the elimination of the low frequency mutation over the course of the experiment. If the latter, selection for the cinnabar eye color was weak in the slow selected line.

Figure 2.8. A *cinnabar C. macellaria* mutant.

Substrate

Early into the experiment the rearing substrate needed to be changed. As handling six different selected lines was quite time consuming, changing from course vermiculite to fine sand drastically dropped the time needed to separating pupae from substrate. With this change, a statistical analysis was needed to test for any notable changes in development time due to the substrate change. By investigating the residuals remaining after accounting for the major contributors to development, I was able to understand that there was likely little influence of substrate on development. I determined the substrate change had almost no effect on development in this case, though substrate was known in other experiments to be an important factor that could influence development in the blow fly *L. sericata* (Tarone and Foran 2006).

Asymmetric Selection Response

The slow selection regime exhibited a much greater response, by 3.5 days, compared to the baseline development times, than the fast selection regime, which sped up by 1.5 days. In addition, heritability from G01-G23 was generally greater in slow developers (11.8-24.7%) compared to fast developers (9.6-17.6%). The higher heritability score in slow selected blow flies means a larger additive genetic component effecting development. There could be a number of reasons for this. Fast development alleles could have been fixed or at high frequencies in the experimental populations studied, thus there was less starting variation to respond to selection. Alternatively, fast

development may be associated with other modes of inheritance, like dominance or epistatic variance, based on the genetic availability remaining after selection.

MANOVA

While the effects evaluated through MANOVA were reported as "genetic" in nature, the resulting temporal autocorrelation could also be due to shared environmental factors, such as drift in incubator temperatures or seasonal changes in the rearing room that led to different maternal egg provisioning. Temporal biases could also cause measurement errors, for example a month where phenotyping was more biased toward estimating longer development times, or possibly due to genetic dependence on previous generations. One interesting feature of the one generation correlation in phenotypes (~0.8) is that the temporal autocorrelation is much greater than the realized heritability in the experiments. This is an argument that suggests there could be environmental or error dimensions to similarities across generations.

Overall

Selection had the strongest effect of all the factors considered in the experiment, explaining 58.6% (η^2) of developmental change in this experiment. Even when the other factors of generation, experimental population, and their respective interactions were significant, they had a modest to low effect size on development time in this study (3.6-18.7%). While other population studies could expand what is known on the development of *C. macellaria* from the genetics determined by its respective experimental population,

this study has produced a deeper understanding of developmental genetics determined by selection.

Biotic factors demonstrated the range of fast and slow development produced by artificial selection articulates the evolutionary genetic potential of these experimental populations. The presence of genetic variability for development has repercussions on forensic entomology with regards to predicting the age of *C. macellaria* collected as evidence, which can be informative of PMI. In the Gallagher study (2010), *L. sericata* age predictions using developmental data (at 16°C) from the wrong population could result in increased error in PMI, up to 13.80%. Blind validation studies are pertinent for aging in PMI studies by determining error rates and accuracy in methods. In the VanLaerhoven (2008) blow fly validation study, a minimum of 10.5% error in PMI estimates was reported. The results from the study conducted here suggest that much of the current error in fly age estimates could be due to genetic discrepancies between experimental and casework samples. The heritability values in this experiment were in line with the current reported error in forensic entomology.

Abiotic factors may have also influenced development over the course of this study. When pupae were counted, damage to pupal casings may occur to some when picked or sifted, either altering or halting development. When counting adults, blow flies were cooled to make them manageable and thermal fluctuations in the colony room could influence the adults of subsequent generations.

From this selection study, the five day range produced after 23 generations of selection established a minimum and maximum for the development of these Texas

experimental populations and an estimate of the maximum error due to developmental variation. After an additional 20 generations of selection by Dr. Jonathan Parrott, there is now a six day difference in development, a modest change from the maximum estimated here. The heritability estimated here lends to our understanding of development and the eventual error in aging estimates that is based on the genetic component. The heritability from G01-G23 established fast-selection h^2 ranged from 9.6-17.6% and slow-selected h^2 ranged from 11.8-24.7% among the experimental populations. These values are in line with expected error due to genetics from the Gallagher study on *L. sericata* developmental variation.

In conclusion, I have demonstrated that there was ample genetic variation for development time in *C. macellaria*, wild flies were more like fast selected flies than slow selected flies, and the majority of genetic variation appears to be an order of magnitude greater within, not between experimental populations. These results have repercussions for our understanding of the evolution and ecology of this species, and show the importance of directing decisions towards studying the genetics of blow fly development. This study demonstrated that it may be possible to quantify potential error from genetic in a manner that can address expectations of uncertainty in estimates of blow fly age in forensic entomology.

CHAPTER III

PHENOTYPIC PLASTICITY AND TRAIT CORRELATIONS OF SELECTED LINES

Introduction

As shown in chapter 2, selection on *Cochliomyia macellaria* development time was shown to result in ~5 days difference in development of wild-derived strains. Faster developing lines demonstrated less selection response than slower developing lines, suggesting that wild fly populations naturally possess more "fast" alleles that determine development time. This chapter considers the evolutionary ecology of the species *C. macellaria*, investigating genetic and environmental effects on life history traits, including correlations and tradeoffs across temperatures among potential fitness components of development, mass, and immature viability. From this research we can extend our current understanding of basic biology of the organisms in diverse environments.

Genetics, environment, and their interaction influence phenotypic traits. Additionally, it is well known that life history traits exhibit correlation with each other, in a complex and environment-dependent manner (Clare and Luckinbill 1985, Via and Lande 1985, Hoffmann and Parsons 1991). For instance, fast development could correlate with smaller adult size (Calder 1984, Atkinson 1994) or increased immature

survival (Prasad et al. 2000, Rose et al. 2004). Since evolutionary fitness reflects the sum total of all possible components of fitness, it is possible for selection to act on one trait to ultimately impact the evolution of other correlated traits as well (Roff 1992). In addition, since selection varies between environments, phenotypes may exhibit differential responses when comparing specific populations under specific selection regimes. The nature of plasticity depends on the natural selection of specific alleles that express a desirable trait for specific environments (Thompson 2005). Because of these correlated responses and environmental particularities, possible impacts of selection on life history traits can result in non-intuitive and unexpected outcomes (Palmer and Dingle 1986, Roff and Mousseau 1987, Harshman and Hoffmann 2000).

Temperature has a powerful effect on insect life history traits, including metabolism and development (Byrd and Castner 2001, Kasson 1999, Davies and Rattcliffe 1994). Temperature is an important factor regulating biochemical activity and it is not surprising to find that it can impact fitness related traits such as body size and development time in insects (Burt 1995), and impact their correlations, including survival and fecundity (Calder 1984, Schmidt-Nielsen 1984). Insects developing at warmer temperatures tend to be smaller (Atkinson 1994). When *D. melanogaster* are reared at lower temperatures, body size variation between males and females is reduced (David et al. 2011). Sexual dimorphism may exist, but overall there is a healthy median size that is best for fitness; extreme sizes can be detrimental (Chown and Gaston 2010). Different populations of the ant, *Temnothorax curvispiosus* (Mayr) (Hymenoptera:Formicidae) adapt to survive in diverse temperatures, although they excel

in cooler environments (Pelini et al. 2012). This is the antithesis of *C. macellaria*, as more blow flies survive in warmer rearing temperatures (Owings et al. 2014).

Each environment is distinct, with varying temperature and ecosystems, therefore organisms must make trade-offs by following environmental cues to determine how to invest time, energy, and nutritional resources to adapt and survive (Hammill et al. 2008); no single phenotype can excel in all environments (Via and Lande 1985, Moran 1992). Ecologists and evolutionary biologists frequently study a variety of adaptive traits and their trade-offs to understand the evolutionary dynamics of adaptation in the wild (Newman 1992, Via et al. 1995, Pigliucci 2001). Calliphoridae demonstrate trade-offs in fitness as body size increases, and development time changes (Roff 1992), which increases the survival risk from parasitism, predation, and ephemeral resource depletion (Nijhout et al. 2010). High larval density also has an effect by reducing parasitism or predation (Rohlfs and Hoffmeister 2003) or reducing body size, fecundity, and longevity due to intraspecific competition (Smith and Wall 1997, Green et al. 2003, Shiao and Yeh 2008). However, blow flies demonstrate a positive correlation of fecundity and body size (Bennettova and Fraenkel 1981, Chapman 1998), as well as mating opportunities (Stoffolano et al. 2000).

For these reasons, organisms must adapt to what the ecology demands of them, balancing between the costs and benefits of phenotypic traits exhibited in particular environments. Life history traits of development time and body size are sometimes under conflicting evolutionary pressures, and trade-offs are often made to alleviate this challenge (Stearns et al. 1991, Davidowitz et al. 2005). Little is known about trait correlations in wild *C. macellaria*, thus this study was developed to evaluate correlated trait responses and plasticity after selection on development time. This chapter investigates the plasticity of development time, total pupal mass and immature viability, all of which are important to evolutionary fitness (Kozlowski 1992, Rosa et al. 2004). It also provides information on diverse correlated life history traits as they relate to wild *C. macellaria* fitness.

Materials and Methods

Experiment 2: Phenotypic Plasticity of the Selected Lines

The last three generations of the fast and slow selected lines of College Station, Longview, and Snook experimental populations were raised in the same conditions as the previous chapter, except lines were exposed to three rearing temperatures. In this experiment, there was a generational difference, first, because the initial Longview line being collected a month prior to the College Station and Snook lines, and second because the faster development lines were six generations ahead of the slower development lines at the start of this experiment. Due to the different generational timing, with fast lines ranging from G27-G30 and slow lines ranging from G21-24, small discrepancies are expected when comparing data to Chapter 2 (especially when evaluating fast selected flies, as only up to G23 was analyzed there). To establish the starting populations for these experiments, the three fast selected and three slow selected lines were split from the 25°C line to produce two additional temperature regimes: 20°C and 30°C. The resulting 18 strains were deposited in their incubators on the same day to allow for direct comparisons. The 25°C and 30°C lines were able to produce their subsequent generations, although due to the extremely slow development rate and low numbers of the 20°C lines, it was necessary to draw eggs from the 25°C colony each generation. The flowchart (Figure 3.1) below describes of the experimental design.

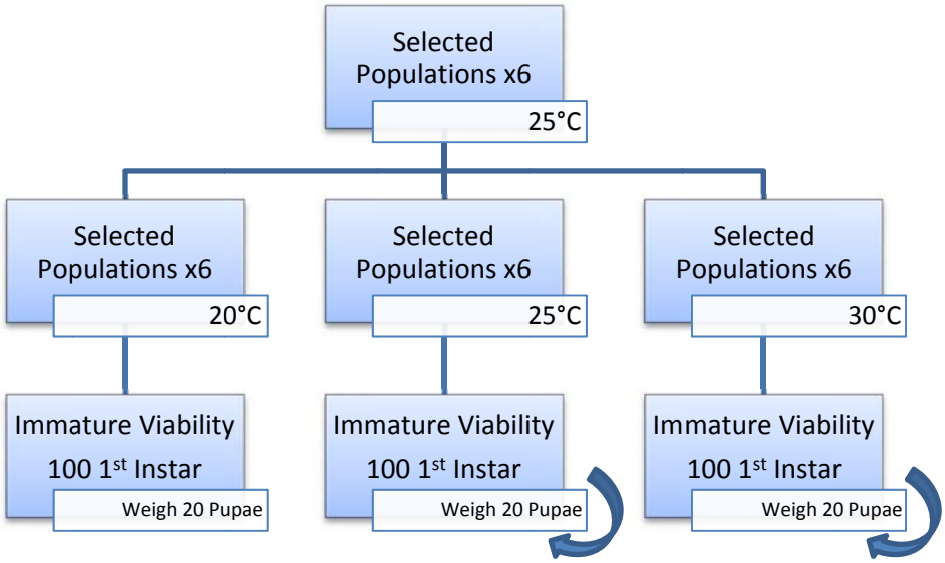


Figure 3.1. Experimental design for phenotypic plasticity of the selected lines. The six selected lines were split for three thermal regimes and simultaneously run for three generations to investigate 18 experimental lines for plasticity. Development was run and logged as before. One hundred first instar larvae are separated to evaluate immature viability and 20 pupae from those 100 were weighed to evaluate the plasticity of mass. Arrows denote that the generations for lines at 25 and 30ºC were continued from their respective lines, while 20ºC lines were pulled from the 25ºC for each generation.

Egg Distribution to Three Thermal Regimes

The egg collection process was repeated as before for each of the six selected strains reared at 25°C and repeated to produce the 20°C and 30°C lines. The collected eggs were gently mixed and distributed among three sets of jars, with one set to each incubator for the respective thermal regimes. The number of jars within each set varied was based on the number of eggs available with approximately 200 eggs per 1 quart

Mason jar. The goal was 1,200 eggs per line for each generation, but varied due reproductive output of the inbred selected lines and low viability at cold temperatures. The 18 sets of rearing jars were labeled according to the respective selection and experimental populations with 18 lines total. The methods described under "Immature Viability" and "Mass" below occur simultaneously.

To ensure ovary maturation for egg collection, blood meals were provided, with approximately 5mL in a paper towel lined petri dish, fed *ad libitum*. Fast lines required 2-3 days of blood meals and slow lines required 3-4 days of blood meals. After the eggs were collected and several had developed into first instar larvae, 200 parents were frozen at -20°C, in two labeled 15mL conical tubes and archived at -80°C for later genetic analysis. When the majority of the larvae pupated, the sand was carefully sifted. Pupae sifted from each of the 18 temperature and selected lines were counted and stored in mosquito breeder cages or quart-size Mason jars in preparation for adult eclosion. Newly eclosed adults were counted and chilled as before to make counting more manageable. Data was logged for each collection at each time point for each of the 18 experimental lines, recording eclosion time, number of males, and number of females. This process was repeated four times a day over the course of the eclosion period, ranging from 2 – 7 days.

The first 200 fast flies and the final 200 slow flies used for breeding the next generation were selected for each of the respective lines in all three thermal regimes. The fast and slow flies from the 25°C rearing went into the original Bug Dorm cages as

before. Flies from the 20°C and 30°C treatments were selected and stored in collapsible rearing cages (BioQuip 1468B, 13" cube with sleeve). All cages were provided distilled water and dried sugar in a petri dish, *ad libitum*. Once the adult flies from each temperature and selection speed were caged, the blow flies started their blood meal. The egg collection process was repeated as described before for each generation. Due to low numbers of the 20°C lines, eggs were pulled from the respective selection regime of the 25°C colony to produce the next generation.

Immature Viability

To prepare eggs for the immature viability study, eggs were collected from the 18 experimental lines, see "Egg Distribution to Three Thermal Regimes" above. Eggs were carefully mixed to reduce batch effect, and approximately 200 eggs from each line were transferred to a separate 3 oz. cup containing bovine liver and wet Kim-wipes. The 3 oz. cups were stored in a pint sized Mason jar covered with a Wypall, sealed with a Mason jar lid ring and labeled. The 18 jars were placed in their respective incubator, six at 20ºC, six at 25ºC and six at 30ºC, to hatch over 48 hours. When first instar larvae were present, 100 were carefully transferred, via wet paint brush, to a rearing Mason jar. The quart sized Mason jar was prepared for rearing just as before: half-filled with sand, topped with a small paper towel, with approximately 50g of bovine liver covered with a Kim-wipe, sealed with a Wypall and a jar lid. The 25°C and 30°C first instar larvae were present and transferred after 24 hours, while the 20°C first instar larvae were present and transferred only after 48 hours, due to the much slower development rate at 20°C. The

100 larvae in the 18 jars growing at the three temperatures were observed for survival over the course of their development. The subsequent pupae were stored in jars or 24 well plates (see "Mass" below), until the blow flies eclosed to record development time, sex, and if the blow flies succeeded to adult stage. The remaining larvae that were not the 100 counted for immature viability were placed into jars separately to record development rate times and sex.

Mass

Once 100 larvae from "Immature Viability" reached the pupal stage, 20 of the 100 pupae were randomly picked and weighed to a tenth of a milligram on an Ohaus Adventurer Pro electronic scale. The pupal were weighed and the weight logged and subsequently maintained individually in a Corning 24 well culture plate (Corning 3524) in their respective incubator to complete their development. In total each emerging adult was logged for egg collection time, pupal mass (mg), eclosion time, and sex.

Statistical Analyses

Statistical analyses were performed with JMP 10.0, which performs a Type III ANOVA. The factors tested in the models were generation, temperature, selection, experimental population, and sex. Datasets based on the mean phenotype of individuals reared from the 18 jars at 20, 25, and 30°C were utilized to test phenotypic plasticity: overall immature development time, overall mass, mass between the sexes, and overall immature viability.

A number of ANOVA statistical models were run to analyze the phenotypic plasticity in this study, evaluating the extent to which each of the factors, or their interactions, explain the variation in the phenotype of interest. Model assessments were organized from highest to lowest AICc (defined below) to show improvement of model quality; the R^2 for each shows how well the model explains the data. The AICc measure reflects the likelihood that a model explains the data better than other models; more likely models have lower AICc scores. An ANOVA was performed on the model with the lowest AICc score using Student's t-tests and Tukey's HSD (Honestly Significant Difference) to determine significant difference among means for each categories ($p < 0.05$). Eta squared (η^2) was calculated, just as in chapter 1, to observe and compare effect size (%) of individual factors and the contribution of each to the ANOVA models fit to the overall phenotypic variation.

To utilize the normal distribution expected from the central limit theorem, the mean phenotypic data from the 18 lines for each of the three generations was used for statistical analysis. Replicate as listed below was to denote "generation" in this chapter. These are not strictly independent replicates, but given that no overall differences were observed between generations in the different selected lines, using generations as replicates simplified the statistical analysis. Population as listed in the following model assessments and ANOVAs refers to three replicate experimental populations. Immature viability was based on the percentage of first instar larvae that succeeded to the adult stage. It should be noted, that while pupal mass was logged for all pupal samples, the statistical analyses used only the data for the viable adults that emerged as those blow flies have a development time and sex for comparison in this study.

Results

Model Assessment: Overall Immature Development Time

The relative contributions of selection, temperature, experimental population, mass, and replicate (generation) to the total phenotypic variation were assessed for the model with the lowest AICc in an effort to describe the overall immature development. Temperature had the highest effect size of the single factors (75.7%), followed by selection (20.2%), these factors and its interaction had one of the lower AICc values (Table 3.1, model 32), but was lowered when population was included into the model. The remaining single factors of mass, experimental population, and replicate (generation) were negligible, each with an effect size under 1%. The combination of temperature, selection, and experimental population had the smallest AICc and offered the most likely model to explain overall immature developmental data (Table 3.1, model 34) of selected *C. macellaria* lines reared at three different temperatures. It is important to note that temperature was the greatest factor in describing immature development time; this effect reflects plasticity remaining in the selected lines. It is equally important to note the significant temperature and selection interaction, while small, still exceeded population and the population interactions. Similarly in chapter 2, several models concerning the sex effect were performed (not shown here) which also showed a low effect on development; therefore, sex was not included from this model assessment on development time.

Table 3.1. Model assessment of phenotypic plasticity for total immature development. Models assessed utilized a single dataset of mean development time per generation of the selected lines, for three generations, among various factors.

Model	Development =	R^2 (%)	AICc
1	Mass + Population + Replicate	1.6	711.9
2	Population + Replicate	0.87	709.6
3	Mass + Replicate	0.67	707.2
4	Mass + Population	1.6	706.7
5	Replicate	0.0086	705.1
6	Population	0.86	704.7
7	Mass	0.67	702.4
8	Selection + Population + Replicate	21.1	699.9
9	Selection + Population + (Selection x Population)	21.7	699.5
10	Selection + Replicate	20.2	695.4
11	Selection + Population	21.1	694.8
12	Selection	20.2	690.6
13	Selection + Replicate + Population + Mass	43.7	684.5
14	Selection + Population + Mass	43.3	679.5
15	Selection + Mass	41.4	676.3
16	Temperature + Population + (Temperature x Population)	76.7	642.8
17	Temperature + Population + Replicate	76.6	637.1
18	Temperature + Replicate	75.7	633.6
19	Temperature + Population	76.6	631.7
20	Temperature	75.7	628.7
21	Temperature + Population + Replicate + Mass	84.9	616.3
22	Temperature + Replicate + Mass	84.3	612.8
23	Temperature + Population + Mass	84.7	611.4
24	Mass + Temperature	84.1	608.4
25	Selection + Temperature + Replicate	95.9	539.6
26	Selection + Temperature + Mass	96.0	536.1
27	Selection + Temperature	95.9	534.5
28	Selection + Temperature + Population + Replicate + Mass	96.9	533.3
29	Selection + Temperature + Population + Replicate	96.8	532.3
30	Selection + Temperature + Population + Mass	96.9	527.4
31	Selection + Temperature + Population	96.8	526.8
32	Temperature + Selection + (Selection x Temperature)	97.7	508.8
33	Temperature + Selection + (Selection x Temperature) + Population	98.6	488.9
34	Temperature + Selection + (Selection x Temp.) + Population + (Population x Selection) + (Population x Selection x Temp.) + (Population x Temp.)	99.4	480.5

Overall variation in immature development was significantly explained ($p < 0.0001$, Table 3.2) by the combination of selection, temperature, and population. From that model, differences in the mean values of fast and slow development rates (selection effects) were significantly different based on the Student's t-test, as were the average development times at each of the three thermal regimes, based on the Tukey's HSD test. From the thermal regime, mean development spanned 3.72 days at 30°C, 5.58 days at 25°C, and 7.86 days at 20°C over the final three generations. The significant population and selection interaction was observed because the slow Snook line developed significantly quicker than the slow College Station and Longview lines (Figure 3.2); none of the fast developing experimental populations were significantly different from each other.

In the previous chapter, the development rate of each selection group was compared at G23. In this chapter, the slow G21-G23 lines were compared against the fast G27-G30 lines as the faster lines had shorter generational periods and were six generations ahead. Comparisons of immature development between chapters exhibit small discrepancies due to those differences.

Table 3.2. ANOVA of phenotypic plasticity for overall development. Model 34 from table 3.1. ANOVA output of development time and the various effects of temperature, selection, and population for the final three generations.

Phenotype	Model	Source	Df	Sum of Squares	Mean Square	F ratio	Prob. > F	η^2 %
	ANOVA	Model	17	1251169.4	73598.2	350.250	<0.0001 *	99.4%
		Error	36	7564.7	210.1			0.6%
Development		Total	53	1258734.1				
	Effect	Temperature (Celsius)	2	953276.15		2268.295	<0.0001 *	75.7%
	Tests	Selection	1	254363.62		1210.503	<0.0001 *	20.2%
		Selection*Temp.	2	22259.29		52.965	<0.0001 *	1.8%
		Population	2	10879.84		25.888	<0.0001 *	0.9%
		Pop.*Selection	2	7435.17		17.692	<0.0001 *	0.6%
R^2=0.994		Pop.*Selection*Temp.	4	1646.32		1.959	0.1218	0.1%
AICc=480.5		Population*Temp.	4	1309.05		1.557	0.2067	0.1%

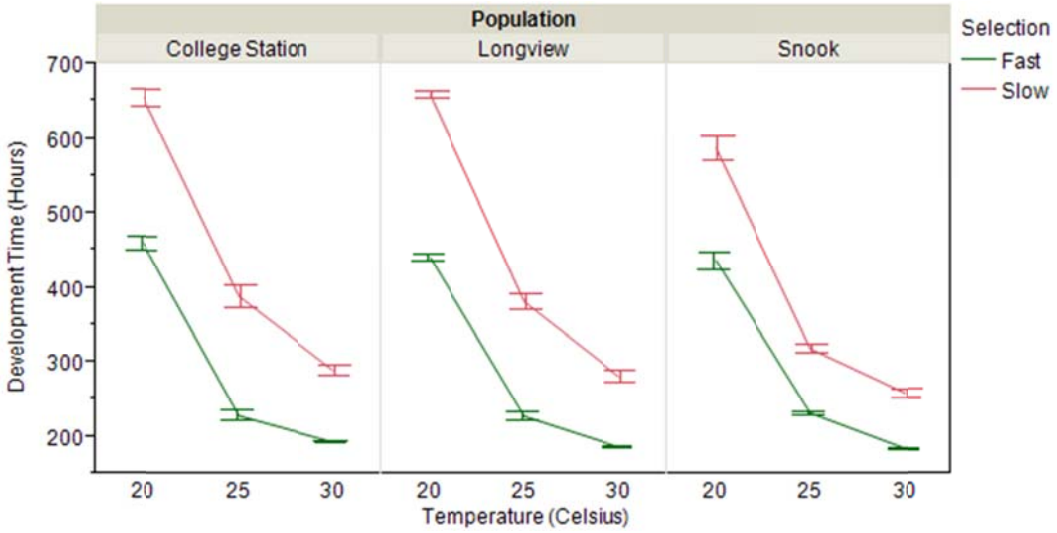


Figure 3.2. Phenotypic plasticity of development for the selected lines. Developmental rate in hours (y-axis) of selected lines with standard error bars, fast in green and slow in red, at three temperatures of the three experimental populations (x-axis).

Model Assessment: Overall Mass of Viable Adults

The factors of selection, temperature, experimental population, and replicate (generation) were assessed for the model with the lowest AICc to describe the overall mass of secondary screwworms that succeeded to the adult stage. Basic ANOVA models were assessed and selection had the highest effect size of the single factors (38.6%), followed by temperature (17.9%) with interaction contributing 10.1% effect on mass of viable adults. The remaining factors of experimental population and replicate (generation) were negligible, with an effect size under 2%. The combination of temperature and selection model explained the majority of variation in overall mass (Table 3.4) of *C. macellaria* under the thermal selection regime.

Table 3.3. Model assessment of overall mass. Models assessed utilized a single dataset of mean mass per generation of the selected lines, for three generations.

Model	Mass =	R^2 (%)	AICc
1	Temperature + Population + (Temperature x Population)	22.2	366.6
2	Population + Replicate	2.4	365.5
3	Population	1.0	362.3
4	Replicate	1.4	362.1
5	Temperature + Population + Replicate	20.3	361
6	Temperature + Population	18.9	356.5
7	Temperature + Replicate	19.3	356.3
8	Temperature	17.9	352.2
9	Selection + Population + Replicate	40.9	342.0
10	Selection + Population	39.6	338.1
11	Selection + Replicate	39.9	337.8
12	Selection + Population + (Selection x Population)	48.7	334.5
13	Selection	38.6	334.2
14	Selection + Temperature + Population + Replicate	58.9	328.2
15	Selection + Temperature + Population	57.5	324.3
16	Selection + Temperature + Replicate	57.8	323.8
17	Selection + Temperature	56.5	320.4
18	Selection + Temperature + (Population x Selection) + (Selection x Temperature) + (Population x Selection x Temperature) + (Population x Temperature) + Population	83.6	316.9
19	Selection + Temperature + (Selection x Temperature)	66.6	311.3

The model that fit overall masses of viable adults for the selected lines at different temperatures was highly significant ($p < 0.0001$, Table 3.4). Selection and temperature effects were both significant ($p < 0.0001$), as was the two-way interaction ($p < 0.0018$). Based on the Student's t-test, slow lines exhibited an average mass of 47.07 mg, with a significant difference from fast lines average of 39.12 mg. For temperatures, mass at 20°C (39.50 mg) was significantly different from the 25°C and 30°C (45.80 mg and 43.98 mg, respectively), according to the Tukey's HSD. The two-way interaction showed a significant difference between slow line masses at 25°C and 30°C, 51.85 and 48.38 mg respectively, and the rest of the other lines; slow 20°C at 40.99 mg, fast 20°C at 38.00 mg, fast 25°C at 39.76 mg, and fast 30°C at 39.59 mg.

Table 3.4. ANOVA for overall mass. Model 19 in table 3.4. ANOVA output on mass (mg) of all blow flies that made it to the adult stage and the effects of selection, temperature, and selection x temperature (°C).

Phenotype	Model	Source	Df	Sum of Squares	Mean Square	F ratio	Prob. > F	η^2 %
	ANOVA	Model	5	1481.419	296.284	19.123	<0.0001 *	66.6%
Mass		Error	48	743.686	15.493			33.4%
		Total	53	2225.105				
	Effect	Selection	1	858.162		55.389	<0.0001 *	38.6%
R^2=0.666	Tests	Temperature (Celsius)	2	398.746		12.868	<0.0001 *	17.9%
AICc=311.3		Selection*Temperature	2	224.512		7.245	0.0018 *	10.1%

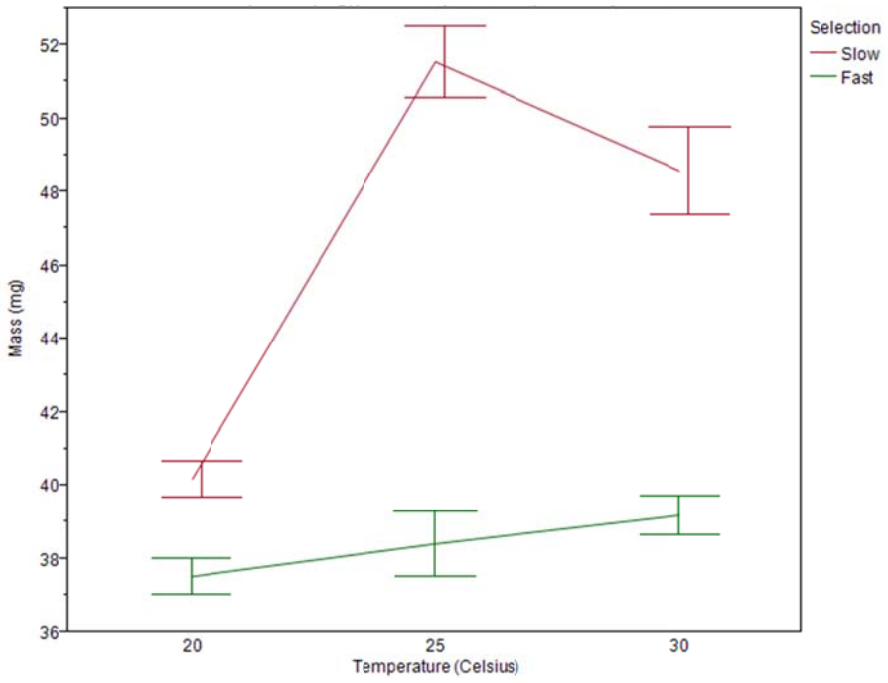


Figure 3.3. Phenotypic plasticity of mass for the selected lines. Mass (mg, y-axis) of selected lines against the three temperatures of all viable adults (x-axis) for the three generations. Each error bar was constructed using 1 standard error from the mean. Tukey's HSD test lists mass of slow at 25°C and 30°C significantly heavier than the other lines ($p < 0.0001$).

Model Assessment: Mass between the Sexes

The factors of selection, temperature, experimental population, and sex were assessed for the model with the lowest AICc to describe the mass between the sexes. A dataset was compiled of both male and female sex to assess influence on mass, separate from the average mass per generation of each line, and therefore sex could not be included with the model assessment "Overall Mass of Viable Adults" above. Basic ANOVA models were assessed, including mixed models with sex as a fixed effect. Selection was the most informative and had the highest effect size of the single factors. Sex was insignificant and exhibited the weakest effect on mass at 1% with an F-test of 1.068 as a single factor or 3.039 when included with selection, temperature, and its interaction model. As sex has an insignificant effect on mass, it was not included from this study.

Model Assessment: Overall Immature Viability

The factors of selection, temperature, experimental population, and replicate (generation) were assessed for the model with the lowest AICc to describe the overall immature viability. Basic ANOVA models were assessed and selection had the highest effect size of the single factors (23.7%), followed by temperature (12.0%). Population had a low effect size of 3.4%, while replicate (generation) was negligible, at 0. 1%. Temperature and selection were the most informative combination to explain overall immature viability. If population and its interactions were added, it could explain additional sources of variation in immature viability and be compared to the simpler selection and temperature combination model. In this case, the model with least factors and lowest AICc was considered best and was evaluated based on the F values in the ANOVA (below). Sex was not known for the unviable blow flies and could not be investigated.

Table 3.5. Model assessment of immature viability. Phenotype described by experimental population, replicate (generation), temperature, and selection.

Model	Immature Viability (%) =	R^2 (%)	AICc
1	Population + Replicate	3.5	469.5
2	Temperature + Population + Replicate	15.4	467.8
3	Selection + Temperature + Population + Replicate	39.2	452.8
4	Replicate	0.1	466.4
5	Population	3.4	464.6
6	Temperature + Replicate	12.1	464.5
7	Temperature + Population	15.3	462.4
8	Temperature	12.0	459.6
9	Selection + Population + Replicate	27.2	456.9
10	Selection + Replicate	23.9	457.4
11	Selection + Population	27.1	451.8
12	Selection + Temperature + Replicate	35.8	450.1
13	Selection	23.7	449.5
14	Selection + Temperature + Population	39.1	447.3
15	Selection + Temperature + Population + (Population x Selection x Temp.) + (Selection x Temp.) + (Population x Temp.) + (Population x Selection)	74.0	445.4
16	Selection + Temperature	35.7	445.0
17	Selection + Temperature + (Selection x Temperature)	42.9	443.9

The mean immature viability was significantly different for the selected lines under the three thermal regimes ($p < 0.0001$, Table 3.6). Selection displayed a significant difference between fast and slow lines ($p < 0.0001$) and the strongest η^2 at 23.7%. Temperature was also significant ($p = 0.0105$), with a η^2 of 12.0%. The two-way interaction of selection x temperature was not significant ($p = 0.0589$). Fast developers exhibited a significantly higher survival rate of 84.7% compared to the 67.9% of slow developers. There was a significant difference in survival of blow flies reared at 30°C and 20°C, 83.7% and 69.1% respectively. The immature viability of 25°C lines at 76.2% were not significantly different from either 20°C or 30°C lines. The Tukey's HSD test for the selection and temperature interaction displayed a lower survival from the slow line at 20°C, compared against all the rest, except for slow at 25°C. Slow developers at 20°C showed 54.8% survival, compared against the slow developers at 30°C and the fast lines at all temperatures (80.6-86.7% survival). Slow developers at 25°C were not significantly different from other groups, at 68.2%.

Table 3.6. ANOVA for overall immature viability. Model 17 in table 3.6. ANOVA output of immature viability and the effects of selection, temperature, and selection x temperature.

Phenotype	Model	Source	Df	Sum of Squares	Mean Square	F ratio	Prob. > F	η^2 %
	ANOVA	Model	5	6889.259	1377.85	7.199	<0.0001 *	42.9%
Immature		Error	48	9186.0	191.37			57.1%
Viability		Total	53	16075.259				
	Effect	Selection	1	3816.963		19.945	<0.0001 *	23.7%
R^2=0.429	Tests	Temperature (Celsius)	2	1921.815		5.021	0.0105 *	12.0%
AICc=443.9		Selection*Temperature	2	1150.482		3.006	0.0589	7.2%

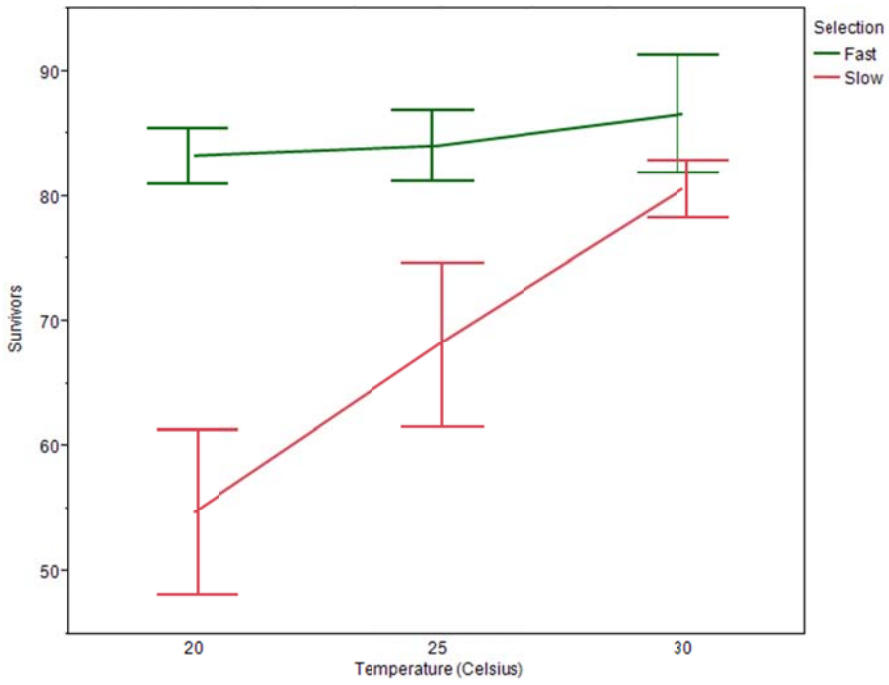


Figure 3.4. Phenotypic plasticity of immature viability for the selected lines. Percentage of survivors (immature viability, y-axis) that made it to adulthood of selected lines at three temperatures for the average of the three experimental populations (x-axis). Each error bar was constructed using 1 standard error from the mean.

Phenotypic Plasticity Summary

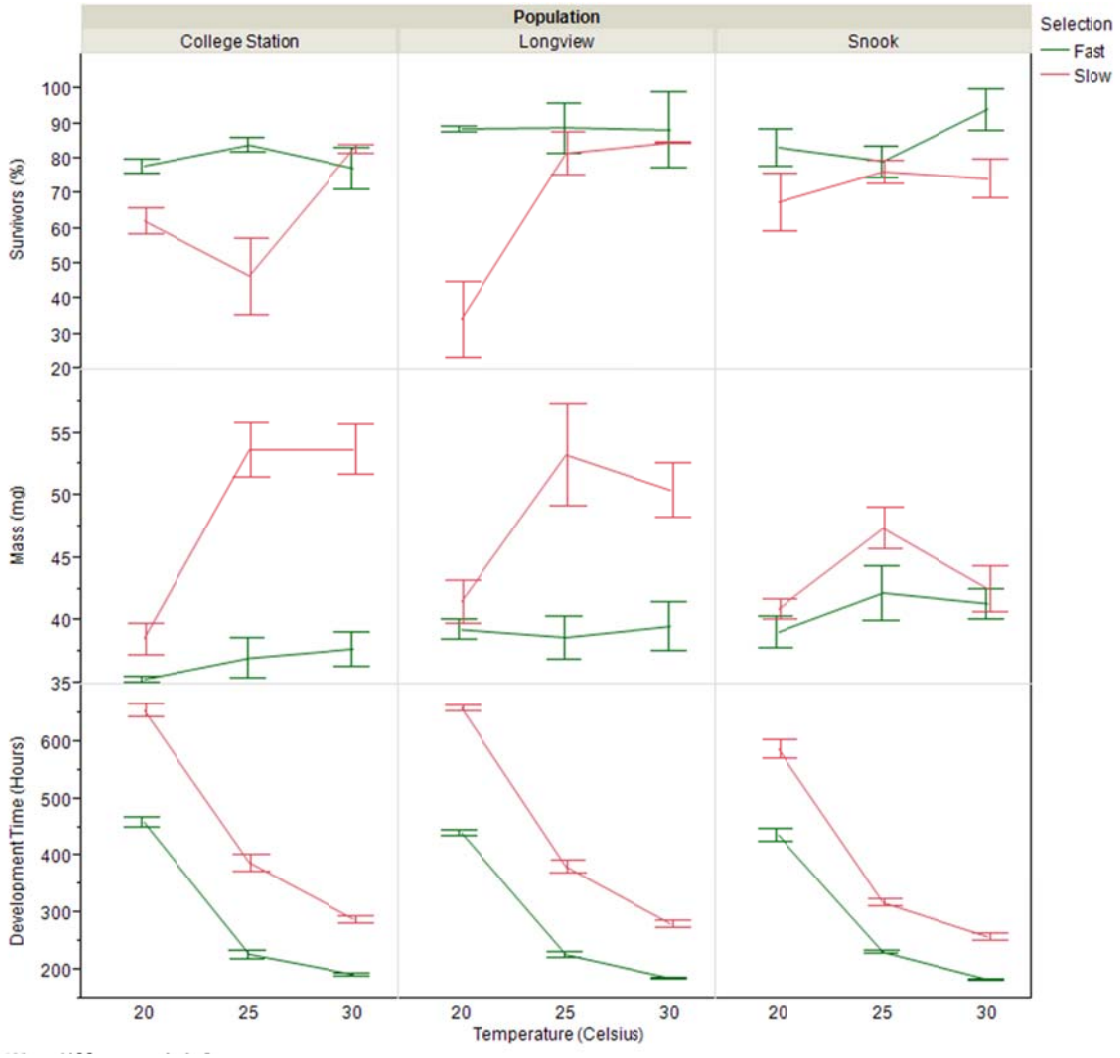


Figure 3.5. Summary of phenotypic plasticity. Three investigated phenotypes (y-axis) at three different temperatures (x-axis). Columns of the three experimental populations were included to display intraspecific variation and influence. Each error bars were constructed using 1 standard error from the mean.

Variance differed among the phenotypes of the selected lines in the three thermal environments (Table 3.7). For development, higher variance was observed among all the slow developing selected lines at all temperatures, compared against the initial G01. Higher variance was also observed at cooler rearing temperatures, 20ºC, even for the fast developing selected lines. The variance for development among the fast selected lines reduced at 25 and 30ºC. For the mass and immature viability phenotypes there were no G01 variances to compare against. The mass variance of the slow selected lines were consistently greater than fast selected experimental populations of the same thermal regimes. The immature viability variance was greater among the slow selected lines at 20 and 25ºC, but not 30ºC.

Table 3.7. Variance in phenotypes for the final three generations of the respective selected lines. Variance from the G01 was included as a baseline for comparison for immature development.

			Slow G21 - G24			Fast G27 - G30		
Phenotype	Experimental Population	G01 25°C	20°C	25°C	30°C	20°C	25°C	30°C
Immature Development	College Station	219.3	2986.7	1425.0	1038.7	417.9	205.2	93.8
	Longview	277.1	2434.1	1350.4	586.3	345.3	187.8	141.4
	Snook	257.9	1486.5	734.7	594.5	496.5	91.5	42.5
	All	344.2	3985.5	2483.7	906.1	497.3	165.6	107.4
Mass	College Station	N/A	36.8	29.1	24.6	15.5	15.5	17.83
	Longview	N/A	19.9	68.0	37.8	11.6	17.0	17.0
	Snook	N/A	20.0	22.2	29.6	13.5	22.0	10.9
	All	N/A	27.7	48.7	50.6	16.7	24.8	18.8
Immature Viability	College Station	N/A	42.3	356.3	4.0	13.0	12.0	101.3
	Longview	N/A	357.3	108.3	0.3	2.3	156.0	357.3
	Snook	N/A	209.3	32.3	94.3	81.3	60.3	105.3
	All	N/A	392.7	390.9	47.8	45.5	74.6	198.8

Discussion

After rigorous and extensive selection, the phenotypic plasticity of the selected lines was investigated for three generations. This investigation focused on the phenotypic response of immature development, mass, and immature viability in three different thermal environments. Temperature had the greatest influence on immature development, with wider variation at cooler temperatures, reducing as the temperature increased. Flies from both the slow and the fast-selected lines also developed quicker at warmer temperatures. Genetic interactions in the selected lines played a greater role on mass and immature viability with heavier mass at the moderate to warm temperatures (25-30°C), and increased lethality at cooler temperatures (20°C). As wild type flies develop at a rate that is closer to that achieved in the fast-selected lines, which survive better than the slow-selected lines that survive poorly at cold temperatures, and produce less mass overall, it could be inferred that large body size and low temperature larval viability were less important to survival in the wild than fast development.

Other factors that could affect development, and have other pleiotropic effects on mass and immature viability may have been missed. Selection was continued in the final three generations; however the 20°C experimental populations had to be continually derived from the 25°C stock lines, due to the much longer development time and considerably lower number of viable adults reared at 20ºC. This last did not eliminate potential selection effects, but did mean that selection was carried out at 25ºC, rather than at 20ºC in the cold-reared flies. It is not possible to know if this difference helps

explain why model assessments in this chapter show that generation (replicate) played an insignificant and negligible η^2 (0.0086) in explaining the phenotypes. It is possible that these last three generations of selection had little effect, since selection over >20 generations had reduced available genetic variation, driving immature development to the genetic extremes prior to the initiation of the experiment. It is also possible that local conditions with the cultures mitigated some of the effects of the rearing temperatures. Rearing temperature was controlled in the three incubators used in the experiment, but it was unknown how much the microclimate of the feeding larvae aggregating around the liver or across locations within incubators may have impacted results. With approximately 200 larvae in one Mason jar feeding on the same food source, temperature was expected to rise and likely affect the phenotypes (Rivers et al. 2011). Even so, microclimatic effects did not fully mitigate the effect of the different rearing temperatures. The rearing temperature showed a significant overall affect, and any additional microclimatic effect was not under investigation in this study. It is possible to conclude that selection in the last three generations was largely ineffective, and any effect of selection was small compared to the environmental effect of temperature and the genetic effect produced by the selection for fast and slow-developing lines.

Phenotypic Plasticity: Immature Development

Temperature was shown to play the strongest role on development, with development times of 3.72 days at 30°C and 7.86 days at 20°C. The temperature effect (environmental effect) was greater than that of selection (genetic effect) in this study for

this trait. Other studies have shown similar results, with temperature influencing development time in within and among species comparisons. Owings et al. (2014) conducted a phenotypic plasticity study on wild type *C. macellaria*, at 21°C and 31°C, and showed quicker development at warmer temperatures. Other factors such as sex, replicate, and experimental population, were negligible in affecting development, just as in the previous chapter. In a different development study on *L. sericata*, sexually dimorphic genome sizes were determined by flow cytometry and utilized to determine sex and compare larval lengths (Picard et al. 2013). Males had a significantly smaller genome (661Mb) than females (726Mb), and males developed faster than females overall. At warmer temperatures, both males and females were shorter and developed faster. From Picard's study, utilizing genome size would be an excellent tool in minimizing error in development-based age estimates for cases involving sex-specific development, but does not seem appropriate for this species given the relatively small influence exhibited by sex in *C. macellaria*. Development of *C. macellaria* collected outside of the lab will respond to a number of environmental variables; the effects of sex and experimental population would be minimal in comparison to temperature and genetic effects in this species. In this study the population effect η^2 was negligible. The ANOVA model that included populations had the lowest AICc, yet population effects contributed, little to explaining development time. Overall, these results are useful for forensic entomologists as temperature is most important to variation in the phenotype and is currently considered by practitioners. However, genetics as exhibited by the selection regimes clearly demonstrates that a considerable proportion of currently

unexplained variation in development time may lie in genetic differences between evidentiary flies and those used to develop reference data sets.

Phenotypic Plasticity: Mass

Size, length or weight, is another commonly utilized age indicator for calliphorid larvae (Byrd and Castner 2001). Genetics and the environment interact to determine how a phenotype is expressed. In this study the genotype (selection) η^2 determined twice as much of the variation in mass as temperature (environment) η^2 (Table 3.5). Changes in development may also be altered by other life history traits via pleiotropy, as noted with slow developers being significantly heavier than fast developers (Figure 3.3). Extended time to develop during immature stages would allow for additional time to consume nutrients, with a consequent gain in mass. Mohr (2012) found similar results where life history trade-offs were made during larval stage development on ephemeral resources. In that study, wild flies appeared to sacrifice survival to develop faster. The results here show slower development associated with lower survival and larger mass, indicating that fast development was more important than larger size for fitness in the wild. As slower development offers additional time to increase in mass, so does the opportunity to be predated upon, impacting their survival.

Mass results varied in this study, just as was reported in the Owings et al. (2014) work. While the temperature-size rule is a widespread framework, with ectotherms growing slower and larger in cooler temperatures (Kingsolver and Huey 2008), it should not be generalized to be a catch-all rule (Angilletta and Dunham 2003). Owings

demonstrated that wild type *C. macellaria* weighed less when reared at 31°C than those reared at 21°C, which is the opposite of what was observed in this study (Figure 3.3). Even though similar populations were used (College Station and Longview) some differences were expected from the wild flies studied by Owens versus the selected blow flies studied here. The average mass in Owing's wild type pupae was approximately 32 mg, and closer to approximately 39 mg mass of the the fast-selected flies, than to the heavier slow-selected developers at approximately 47 mg. This demonstrates parallels to the previous chapter concerning development time that shows the wild type G01 blow flies were closer in development and mass to fast developers. This finding also supports the need for genetic information on mass of forensically important blow flies, as measures of size are common age indicators. With a wider variation on mass within this study, this could suggest an increase in error as well, and should be further explored.

Phenotypic Plasticity: Immature Viability

The genetics driven by selection (as measured by η^2) explained the most variation in immature viability, and explained twice as much as the environmental effect of temperature η^2 (Table 3.7). Further, while population explains a significant portion of variation in development rate, it did little to explain immature viability. And yet, while selection and temperature explain the vast majority of the variation in immature viability, the small but significant population effect shows that location can influence the average (Figure 3.5).

These Texas experimental populations could experience temperatures ranging from 21-35°C over the course of a year. It is therefore understandable to find fewer viable adults at 20°C in populations adapted to the warmer weather commonly found in Texas. Other studies have shown that *C. macellaria* possess genetic strategies to survive in extreme conditions and develop through their immature stages, in spite of starvation (Mohr 2012) or thermal stress (Owing et al. 2014). In this study, selection for fast and slow development has altered the genotype. As was seen with the phenotypes development and mass, fast developing blow flies have a higher probability of reaching the adult stage. This is particularly true in Texas and other southern states, where *C. rufifacies* predate on *C. macellaria* at ephemeral resources, influencing thereby each other's development, with that influence extending to effects from *C. rufifacies* excretions (Flores 2013). Predation by *C. rufifacies* potentially acts as a natural selective pressure on *C. macellaria* to be primary colonizers and develop quickly. This ecological interaction with other species alters the community dynamic and reduces fitness as more carrion feeders come to feed on the biomass (Brundage 2011).

Overall

This assessment of life history trait responses to selection on development time demonstrated several important principles. First, several other ecologically, evolutionarily, and forensically relevant phenotypes evolved coincident with selection on development time. Second, these pleiotropic responses manifested differently at various temperatures. These results have important consequences for the biological

impacts of *C. macellaria*. From an ecological perspective, the rate and amount of biomass that can be removed from a carcass by this species is directly dependent on all of the life history traits studied. Clearly, different selection regimes have the potential to impact in different ways the rate and extent of carcass is decomposition. From an evolutionary perspective, wild flies are more similar to fast selected lines, suggesting that the phenotypic and thermal responses of the fast selection regime are likely more important to fitness of wild flies. These results also suggest that fast development, small size, a limited response of size to temperature, and larval viability at around 20°C were all important to survival of the species. From a forensic perspective, the relative importance of significant correlates of forensically important traits speaks to several aspects of forensic entomology error. Temperature was the most important factor to consider when evaluating variation in development time. However, there was ample opportunity for interaction with genotype, and genotype can help explain the variance in development time not explained by temperature itself. This means that, while forensic entomologists have picked the most important factor to study, their neglect of genetics likely explains much of the current error in developmental progression based on estimates of insect age.

Given what is known about error in these types of estimates and what was observed in this thesis, genetics is likely to be a major component of error in the system. It is essential to note that thermal responses of body size – another trait used to predict larval age, was less impacted by temperature than by genetic factors. Thus, given that genetic factors are rarely considered in forensic entomology, estimates of age based on

body size are the most likely to be associated with high error, until mechanisms for evaluating genetics are incorporated into the field. Caution should be imposed when evaluating these traits (for this species) as a predictor of age. Taken together, this study has provided evidence for ample genetic variation in *C. macellaria* life history traits, including interactions with temperature, and has shown that this variation is important for applications of the biology of this species that range from forensics to carrion ecology.

CHAPTER IV

CONCLUSION

Cochliomyia macellaria exhibited considerable genetic and phenotypic variation in immature development time, mass, and immature viability. Selection for the phenotypic extremes of fast and slow development demonstrated the range of possible error in aging blow flies, including the existence of greater additive genetic variation for slow development than for fast. Lower heritability of fast development suggests little change in the wild blow flies, as they adapt to their local environment, and possibly as they adapt to develop rapidly to both avoid predation and to preempt competition with other carrion species on ephemeral resources. Sex and populations had a negligible effect on most life history traits. However, the population effect was significant for development, indicating the existence of between group genetic variation.This was most notably observed with the Snook slow line developmental plateau. Observed intraspecific variation in development also demonstrated the potential errors that could be made when making aging inferences from non-local datasets.

The phenotypic plasticity of the selected lines correlated with the expected genetic response to selection, genetics differences between populations, the response to the different thermal environments, and the intrinsic interactions. Warmer temperatures resulted in faster development, and were consistent across all of the selected lines, as is common among ectotherms. Cooler temperatures resulted in increased variation for

development and mass among all the lines, as well as a higher lethality among the slow developing lines. The genetic variation and phenotypic plasticity of these life history traits stresses how importantly ecology impacts an organism and influences how it thrives in that environment. Because ecology affects the evolutionary biology over long periods of time, gradually modifying genetic variability as a species adapts. This variability change can bring with it additional inherent error when making age estimates, and this error is an important aspect of inferences made for death investigations and later PMI.

My selection research provides data on developmental extremes and its importance in the fields of evolutionary biology, ecology, and forensic entomology. Given the inherent variability found in nature, as demonstrated through selection, aging blow flies should be considered using population data local to evidentiary samples. Even acknowledging that a selection experiment would be incredibly difficult to run in the field due to a fluctuating ecology on a sample set, the knowledge gained from that experiment is needed in order to truly represent the local population. The data from this research, along with previous intraspecific developmental research done with *L. sericata* (Gallagher et al. 2010, Tarone et al. 2007) and *C. macellaria* (Byrd and Butler 1996, Boatright and Tomberlin 2010, and Owings et al. 2014) further supports efforts in scoring age in forensically important blow flies. Mohr's investigations (2012) showed that starved larval *C. macellaria* attempt to develop at the same rate as their fed counterparts, trading off development and mass for survival, often failing to reach critical mass to become a viable adult. As immature fly age is determined by size and

development rate, these effects on the components of life history traits of the secondary screwworm add to the possible error, and especially so given innate pleiotropic connections of development, mass, and survival. In the Owing et al. (2014) investigation, faster development and higher survival at warmer temperatures was observed, and further supported by "hotter is better" (Kingsolver and Huey 2008). By exploring evolutionary ecology concepts that relate the base biology of life history traits to the environment and blow flies' biology, our understanding expands on distribution of error associated with PMI estimates. This innate error is characteristic to a species across regions; and as we learn more about this error distribution, we can glean better age estimates.

Over the course of this study, immature *C. macellaria* were reared in a controlled environment that could alter development and other life history traits. Incubation chambers managed temperature, relative humidity, and diurnal lighting to remove and/or limit common variables. Studies have shown that maggots aggregating on nutrient sources generate metabolic heat, raising the temperature of the microclimate, affecting development (Goodbrod and Goff 1990, Campobasso et al. 2001, Higley and Haskell 2001). As the humidity increases, the moisture of food makes it easier to process and develop faster (Tarone and Foran 2006, Mohr 2012). Other studies have shown that differences in the food source itself will generate different development rates (Kaneshrajah and Turner 2004, Clark et al. 2006, Boatright and Tomberlin 2010). In the laboratory, the light cycle controls the activity associated with the daylight hours, creating a common level of sunlight throughout. Yet, while blow flies are commonly

active during the day, one study showed nocturnal activity was present under artificial lighting at the onset of a low atmospheric pressure system in the warmer spring and summer months (Kirkpatrick and Olson 2007). Therefore any findings in this selection experiment concerning development, mass, or survival, are only in context of the methods used to attain the data. The lab itself, independent of the incubation chamber, offers its own variables that could influence development and selection. The temperature in the adult rearing room, while controlled, also had minor variations possibly influencing development of the next generation.

The F.L.I.E.S. facility that housed these experiments also houses a number of dipteran species, notably, *C. rufifaces* (hairy maggot) that are known predators of larval *C. macellaria*. The hairy maggot has been shown to affect secondary screwworm numbers and development when feeding on the same food sources (Wells and Greenberg 1992). Previous studies conducted in the F.L.I.E.S. facility at Texas A&M have shown that close interactions with *C. ruffifacies* affects development and survival, of the secondary screwworm. Flores (2013), demonstrated a predation independent effect from excretions of the hairy maggot affected the secondary screwworm, increasing development time and larval body size. And, while *C. rufifaces* were not reared in the same incubation chambers, jars, or cages, over the course of the selection experiment this interaction may still influence the measured life history values. Another variable to consider is that the College Station and Snook starting lines were collected miles away from the lab to ensure wild type flies. And yet, due to the high concentration of lab flies that inevitably escape from experiments in the F.L.I.E.S. facility, it could be possible to

collect in the wild descendants of lab reared flies. How far this effect extends is poorly known. Few studies have tested the range of flight of calliphorids. One study in South Africa showed blow flies traveling up to 2.20-2.35 kilometers per day (Braack and Retief 1986); and data from another study suggested that some blow fly species may travel up to 20 kilometers in a single day to find ephemeral resources (Greenberg 1991). As data from this experiment derives from the base populations at the time of collection, any later blow fly collections from the same location could display subtle to drastic changes in genetic variation.

For this research to progress, additional molecular genetic work is necessary to identify genetic markers related to fast and slow development. By quantifying and comparing development of adult samples from early and late generations, developmental alleles can be identified that will lead to further applications. The work of Dr. Tarone and Dr. Sze at Texas A&M University, and that of Dr. Picard at Purdue University, is directed toward sequencing of the genome and transcriptome and this information about the genome is expanding our knowledge of the genes involved in regulating development of *C. macellaria*, and these results are being validated against other selected lines and wild type blow flies. As more sequencing data is compiled, from model to novel insects, a comparison of development in this and other sequenced species offers the opportunity to compare genes and regulation of genes across organisms, and especially compare genes and gene regulation of forensically important species. By expanding what we know of the basic biology of *Cochliomyia macellaria* development,

we take an essential step toward advancing our understanding of blow fly ecology, evolutionary biology, and forensic entomology.

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APPENDIX A

CALCULATING HERITABILITY

Below is an example using the first six generations of the slow College Station line. With each new generation a selection differential is produced. This difference is cumulatively added over generations to the produce the selection sigma differential, which represents the x-axis. The selection sigma differential is plotted against the mean development time, the y-axis, to form a line. Drift can pull the bred hours away from the desired selected direction. Regardless of the direction the difference (absolute value) is added to the sigma selection.

Table A.1. Heritability example. College Station slow selected line example using the first six generations.

	Response (y)			Selection (X)
Gen	All (hours)	Bred (hours)	Selection Differential	Sigma Selection
1	259.311	277.500	18.189	18.189
2	305.004	322.654	17.650	35.839
3	284.021	313.300	29.279	65.118
4	301.720	316.520	14.800	79.918
5	306.664	323.961	17.297	97.215
6	319.391	346.141	26.750	123.965

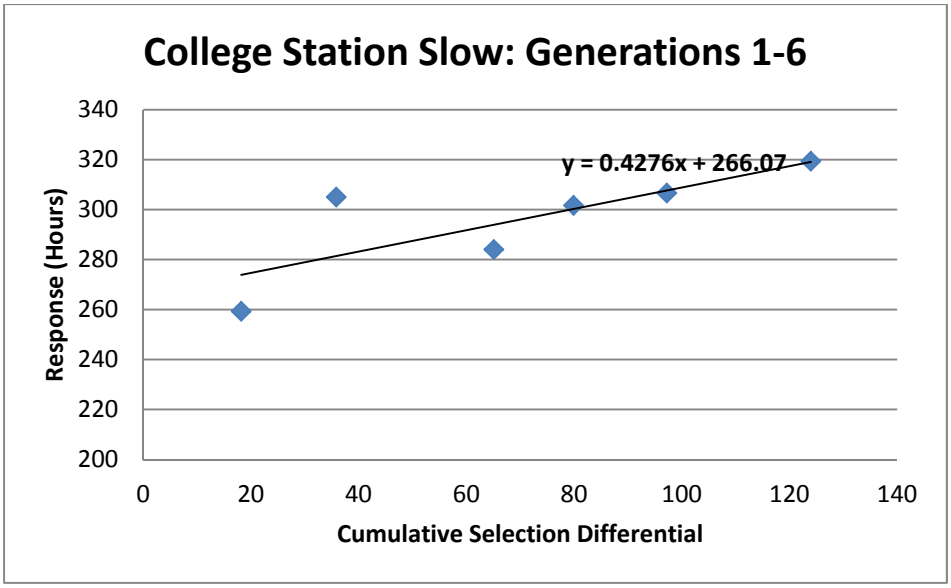

Figure A.1. Heritability example. Line of heritability (slope) for the first six generations of the College Station slow selected line.

Utilizing this data the slope of the line is 0.4276, making the heritability (h^2) of the College Station slow line after six generation $h^2 = .4276$ or 42.8%. As noted previously, the h^2 score can change over time, and may decrease in later generations as selection acts to eliminate additive genetic variation.