EFFECTS OF TEMPERATURE AND TISSUE TYPE ON THE DEVELOPMENT OF *MEGASELIA SCALARIS* (LOEW) (DIPTERA: PHORIDAE)

A Thesis

by

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ABSTRACT

The scuttle fly, *Megaselia scalaris* (Loew), is a Dipteran from the Phoridae family of medical, veterinary, and forensic importance. In the case of the latter, *M. scalaris* is commonly associated with indoor death scenes and its larvae are useful in determining time of colonization (TOC). This is the first developmental study on the effects of different temperatures and tissues from two different vertebrate species on the growth rate and larval length of *M. scalaris*, and consequently, on estimated TOC. A validation study of these data was also conducted. Immature *M. scalaris* were reared on either bovine or porcine *biceps femoris* at 24°C, 28°C, and 32°C. Temperature significantly impacted immature development including egg hatch, development from hatch to pupa, and from pupa to adult. From egg to hatch, development had a growth rate difference of 32.1% from 24°C to 28°C, 13.9% from 28°C to 32°C, and 45.5% from 24°C to 32°C. Development of larva to pupation displayed similar results with differences of 30.3% between 24°C and 28°C, 15.4% between 28°C and 32°C, and 45.2% between 24°C and 32°C. Development from pupation to adult emergence, likewise, displayed a 44.4% difference from 24°C and 28°C, 7.3% from 28°C to 32°C, and 51.2% from 24°C to 32°C. From oviposition to adult emergence, *M. scalaris* needed approximately 32.7% more hours to complete development when reared at 24°C than 28°C, 8.5% when reared on 28°C rather than 32°C, and 38.4% more time when reared on 24°C over 32°C. Tissue type did not significantly impact development.

A preliminary validation study was conducted within four different indoor environments (two different attics, a closet, and a shower) spanning two different buildings. Utilizing minimum and maximum lengths, time of colonization estimates were

underestimated in all instances. In all cases, the range generated encompassed the actual time of colonization. On average, time of colonization estimates when using minimum length were underestimated by 26%, but overestimated by only 1.4% when using maximum development range. Data gathered from this research could be useful when estimating a time of colonization of decomposing vertebrate remains. Future research will need to examine development for each instar in order to increase precision of such estimates.

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TABLE OF CONTENTS

Page

LIST OF FIGURES

FIGURE Page

LIST OF TABLES

CHAPTER I

INTRODUCTION OF LITERATURE IN FORENSIC ENTOMOLOGY: HISTORY AND BEYOND

Forensic entomology is the use of insects and related arthropods for judicial or investigative means. The field dates back to Sung T'zu's "Washing Away of Wrongs" (also referred to as "Collected Cases of Injustice Rectified") being published during the thirteenth century (Catts and Goff 1992). However, forensic entomology did not gain much momentum until the 1800s. Bergeret (1855) submitted the first forensic entomology case report estimating a postmortem interval (PMI), Reinhard (1882) exhumed bodies from Saxony, Germany and utilized phorids (Diptera: Phoridae) associated with the remains to report the first systematic study in the field with recorded methods, controls, and analysis. Later, Megnin (1894) also published his famous work "La Faune des Cadavres" (Benecke 2001) documenting insect succession associated with human decompositional states.

Today, forensic entomology is globally established. This solidification as a sound science is in part due to professional organizations that are forensic entomology-specific. These organizations include, but are not limited to, the European Association of Forensic Entomology established in 2002, and the North American Forensic Entomology Association established soon after in 2005. Forensic entomologists are also members of the Entomological Society of America and the American Academy of Forensic Sciences. Forensic entomologists in North America are certified through the American Board of Forensic Entomology. Presently, there are 17 board-certified forensic entomologists in North America (Table 1).

Table 1. ABFE. List of individuals certified as diplomates and members of the American Board of Forensic Entomology in North America.

Diplomates		
Gail Anderson, PhD Director of Undergraduate Programs School of Criminology Co-Director, Centre for Forensic Research Simon Fraser University Vancouver, Canada	**Jason Byrd, PhD** Associate Professor in the Department of Pathology University of Florida Gainesville, Florida, USA	**Val Cervenka, MS** Private Forensic Entomology Consultant Saint Paul, Minnesota, USA
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Applications

In general, forensic entomology has expanded significantly and can be categorized into three separate sub-disciplines: 1) urban, 2) stored-product, and 3) medico-legal, each serving critical needs of forensic entomology.

Urban

Forensic entomology within the urban field tends to involve pest infestations within human dwellings. Legal matters between individuals and landlords or exterminators, including pesticide treatment management and basic quality of life concerns are typically handled in this field (Catts and Goff 1992).

Controlling insect infestations are typically at the forefront of urban entomology, not only to control property damage, but for public health as well. Cockroach (Blatteria) allergens, for example, have been known since Bernton and Brown (1964) reported positive skin test responses in 332 allergy clinic patients out of 755 living in New York, USA. Fifteen years later, cockroaches were correlated with allergic asthma when 33 of 46 asthmatic subjects were subjected to cockroach antigen and suffered skin reactions. Out of these individuals, 91% (30) suffered immediate asthmatic reaction in a bronchial challenge, and 48% (16) displayed a late asthmatic reaction (Kang et al. 1979).

As previously stated, however, property damage by insects can result in a heavy toll when not properly managed. Estimated economic losses due to termite (Isoptera) activity, presented in Table 2 (Ghaly and Edwards 2011), shows the United States of America (USA) as one of the top "losers" in termite damage at $1 billion (Lewis 2008). Interestingly enough, Japan, a country less than 4% the size of the USA, is second with $800 million in losses.

Such an impact in Japan is thought to be due to the high number of wood structures in the country when compared to other included nations (Verma et al. 2009).

Table 2. Estimated Ecological Cost of Termite Related Damage per Year (Ghaly and Edwards 2011).

Country	Number of Termite Species	Costs Associated with Termite Activity (US $106/year)	Reference
Malaysia	NA	8-10	Verma et al. 2009
India	NA	35.12	Verma et al. 2009
Australia	NA	100	Scholz et al. 2010
China	482	300-375	GEI 2005
Japan	21	800	Verma et al. 2009
USA	50	1,000	Lewis 2008

Non-endemic species cause an estimated $137 billion in damage per year (Pimental et al. 2000). Recently, the Emerald Ash Borer (Coleoptera: Buprestidae) has been making an economic impact in the United States as well. First identified throughout the Detroit, MI, USA metropolitan area in July 2002, this invasive species from China has the potential to kill 23 million ash trees over the next 25 years, causing more than $7 billion worth of damage and replacement costs (Becker 2006).

Incidentals, insects found on carrion but are not commonly associated with or used primarily as entomological evidence, can also be of importance. Most common incidentals include ants, wasps, beetles, and mites. This is especially true when the arthropods in question are very abundant in storage situations, as their association with carrion could simply be a coincidence (O'Connor 2009).

However, what were once considered incidentals have recently begun to cross the barrier into forensic importance. Mites from the infraorder Astigmata effectively merge all

three types of forensic cases (urban, medico-legal, and stored product), as they can be found in any and all of these types of cases (Catts and Goff 1992). Having a phoretic association with insects allows these mites to disperse effectively and be collected in a large variety of environments.

Aforementioned wasps, as well, have recently been taking the lime light in forensic importance. Parasitic wasps in particular are considered the most widespread biotic factor affecting the growth and survival of insects (Turchetto and Vanin 2004). One particular case focused on the parasitoids *Nasonia vitripennis* (Ashmead) (Hymentoptera: Pteromalidae) and T*achinaephagous zealandicus* (Ashmead) (Hymentoptera: Encyrtidae) found parasitising a necrophagous fly population on the body of a young woman. Consequently, the larval population displayed severely shortened survival rates and increased development time.

Stored-Product

Infestations of commercially distributed food or livestock feed by insects or related arthropods fall with the stored-product group. Stored-product infestation investigations may involve, but are not limited to, surveying the food manufacturer, the company used for storage of said product, the transportation company, and the distributor to determine the point of infestation. The forensic entomologist commonly serves as an expert witness during criminal and civil proceedings involving food contamination (Byrd and Castner 2010).

Typically, a local, state, or federal authority will monitor food quality via inspections. In the USA, the US Food and Drug Association complete such inspections. Most importantly, this field is starting to expand thanks to the passing of the Food Safety Modernization Act. This legislation calls for food facilities to self-evaluate any safety hazards in their operations and creates plans to combat these deficiencies. This new ability

holds the food companies directly responsible for any contamination, insect or otherwise, and allows the company to be prosecuted accordingly (FDA 2011).

A number of cases involving insects in food have been highly publicized. A recent seizure in Virginia, USA closed two food products manufacturers for failing inspections due to widespread rodent and insect activity and structural defects reported with no actions taken by the companies to alleviate the situation (FDA 2013). Outside of the US, a recent case was investigated in Malaysia for legal implication. Third instar *Megaselia scalaris* (Leow) (Diptera: Phoridae) were found occupying the inline liquid cream yeast filter used for pipeline circulation in a bread production factory. The case in question was whether the blame fell upon the yeast production industry or the bread producer industry. As yeast is stored at or below 6°C, specimens were sent to Nazni et al. (2011) to test whether or not the *M. scalaris* could not only be reared at 6°C, but reared off of liquid yeast media. At room temperature, *M. scalaris* from egg to adult in liquid yeast media was 14-16 days. At the lower temperature, however, not only could eggs not hatch, but even starting the experiment with first instar larvae saw only 8% survival rate for a maximum of three days before death, indicating the third instar larvae did not come from the stored yeast (Nazni et al. 2011).

Medico-Legal

Entomologists in the medico-legal field are generally asked to analyze "insect evidence" from both living (e.g., myiasis) and deceased humans or companion animals colonized by arthropods (Anderson 2012). Specialists in this group can utilize insect evidence to determine crucial facts for many criminal or civil investigations including postmortem body movement, abuse or neglect, Deoxyribonucleic acid (DNA) acquisition, toxicology

preservation, and time of colonization (Boatright and Tomberlin 2010). Typically, if a body is moved postmortem, eggs or even larvae may be left behind. This includes movement from a room to vehicle, a vehicle to an outdoor environment, or any combination in between (Picard and Wells 2012). Currently, there are two methods in which to accurately determine if a body was moved postmortem utilizing insect evidence.

One method coincides with DNA acquisition and involves the use of genetic analysis of insect gut contents. Human and insect mitochondrial DNA (mtDNA) is extracted from collected maggots at a suspected site where the body was moved (Wells and Stevens 2001). This method can be difficult, however, as the tissue is digested rapidly within the larvae following the time of collection (Campobasso et al. 2005). This creates a very strict time limit between which a maggot is found and submitted as insect evidence, and the gut contents are extracted. The second method proposed calls for genetic tests of "siblingship," a direct relationship of eggs from the same mother in the same batch, between larvae of the same relative developmental stage collected between one location and another. In theory, confirmation of full sibling larvae between two sites would strongly suggest a body was moved postmortem (Picard and Wells 2012). Downfalls of this method include the analysis of a high number of larvae before finding siblings. Furthermore, radical differences in the two environments could affect the development rate of the two siblings in question, decreasing the chance of discovering two or more maggots from the same brood. Despite these obstacles, however, this method could still serve as a viable alternative when mtDNA extraction is unavailable or impossible.

Often, one associates forensic entomologists with assisting with investigations of postmortem remains. Though this may be the case most of the time, forensic entomologists

are also asked to assess insect evidence associated with living individuals during investigations of abuse and neglect (Anderson and Huitson 2004). Myiasis can be caused by self-neglect, as seen in a case involving Phoridae (Diptera) infestation of a morbidly obese man who failed to return for bandage cleaning checkups, and would typically not be criminal in nature (Huntington et al. 2008). Neglect of by a caretaker, however, such as an infant (Benecke and Lessig 2001) or the elderly (Benecke et al. 2004) presents a majority of the cases a forensic scientist may be asked to advise upon. One case in particular involved the death of a mother neglected by her son, who was her primary caregiver. Pressure marks on the side of the chin and chest suggested the two areas were in contact with each other for a prolonged period of time. Furthermore, the presence of *Fannia canicularis* (Linnaeus) (Diptera: Fanniidae) larvae suggested feces and urine were present on the skin, and as the skin was not fed on by the larvae and the eyes were still intact, a conclusion was made that a wound on her foot was likely inhabited pre-mortem. Otherwise, the natural orifices and wound would have been colonized at a similar rate (Benecke et al. 2004).

Entomotoxicology

The use of insects for detecting the presence of drugs and toxins in decomposing tissues, has gained recognition globally (Introna et al. 2001). Along with analyzing for the presence of other chemicals that were likely in the body at time of death, such information has proven useful when estimating a PMI (Goff and Lord 1994).

Skeletal remains possess little to no tissue. Thus, insects present on the remains are useful for toxicological analysis since they harbor toxins within their body. This method of identification was first used in 1980 when a 22 year old, white female committed suicide in a creek bed. There was complete absence of suitable tissue specimens for a toxicological

analysis, and maggots were used in the place of organs. Within the insect evidence, concentrations of phenobarbital up to 100 μg/g were identified (Beyer et al. 1980). It was from here that the theory of drug and toxin identification through maggots was brought to light.

Many cases involving severely decomposed remains utilize associated larvae as a source for toxicological screening. Along with being more abundant, insects are also easily sampled, utilizing the same process one would use to analyze toxins in human tissue (Kintz et al. 1994). Furthermore, "live" samples are more suitable for toxicological analyses, such as chromatography and fluorescence polarization immunoassays (FPIA) (Kintz et al. 1990a), with liquid chromatography in particular being more sensitive to insects than human tissue (Kintz et al. 1990b).

Knowing the presence of such materials in the tissue is critical as these chemicals or toxins impact the growth rates of arthropods collected from the remains. For example, the effects of cocaine on the developmental rate of *Chrysomya albiceps* (Wiedemann) (Diptera: Calliphoridae) and *Chrysomya putoria* (Wiedemann) (Diptera: Calliphoridae) were determined. For *C. putoria*, larvae fed on liver treated with cocaine weighed twice as much as the control larvae. Furthermore, both species completed their overall life cycle 60 hours faster than those in the control (Carvalho et al. 2012). Such differences need to be accommodated when estimating PMI, and entomotoxicology aids in this process.

Narcotics are now known to not only increase development, but in some instances retard growth rate as well. In the case of *Boettcherisca peregrina* (Robineau-Desvoidy) (Diptera: Sarcophagidae) exposed to decaying rabbits overdosed on heroin (Goff et al. 1991) or cocaine (Goff et al. 1989), the larvae were significantly larger in size when fed tissue

containing heroin compared to the control, and the treated maggots remained in pupation longer. This treatment effect resulted in an overall development time within exposed larvae 9 to 42 h more than those in the control (Goff et al. 1989, Goff et al. 1991). Similar deficits in growth rate were observed for *Lucilia sericata* (Meigen) (Diptera: Calliphoridae) when larvae were fed tissue containing morphine (i.e., PMI estimates were reduced by as much as 24 h (Bourel et al. 1999)).

As well, methylenedioxymethamphetamine, or "ecstasy," displayed a significant impact on the development of *Parasarcophaga ruficornis* (Liopygia) (Diptera: Sarcohagidae) with concentrations of 67 mg/g (twice the lethal dose) by up to 25 h when compared to the control. On the other hand, larval mortality is greatly decreased to 2.5% when feeding on a corpse with this extreme dosage, compared to 36.5% in untreated tissue, 43% in 11 mg/g, and 20% in 22.5 mg/g.

Insecticides, such as organophosphates, as well, can be detected in arthropod larvae. In a case involving a 58-year-old male, suicide by the ingestion of malathion was identified as cause of death utilizing gas chromatography. Though malathion was detected in the fat tissue and noted in the gastric contents of the decedent, the insecticide was not detected in the blood, urine, or chest cavity fluid. *Chrysomya megacephala* (Fabricius) (Diptera: Calliphoridae) and *Chrysomya rufifacies* (Macquart) (Calliphoridae), two Diptera commonly associated with decomposing remains on Oahu, Hawaii, USA were submitted for gas chromatography as well. The pooled larvae of 0.28 g tested positive for 2050 µg/g of malathion (Gunatilake and Goff 1989). Likewise, parathion, a far more toxic organophosphate created in the 1940s and distributed as an insecticide and acaricide, was extracted using high performance liquid chromatography (HPLC) from 10 specimens of

Diptera, Coleoptera, Hymenoptera, Isopoda, and Acari in various stages of development and from remains in all stages of decomposition (Wolff et al. 2004).

This was not the first time toxins were successfully screened from insects other than dipteran species associated with decomposing vertebrate remains. Colonies of *Dermestes frischi* (Kugelann) (Coleoptera: Dermestidae) and *Thanatophilus sinuatus* (Fabricius) (Coleoptera: Silphidae), two necrophagous beetles, were reared on rabbit carcasses and minced beef laced with morphine hydrochloride at levels of 0, 10, 20, and 40 mg/h, and 0, 1000, 2500, and 5000 ng/g respectively. Though morphine was detected in all life stages of *D. frischi*, the toxin was absent in the pupal stage of *T. sinuatus*, and present in all others.

Lastly, in a particularly out-of-the-box theoretical spark, the effects of gunshot residue was examined in *Calliphora dubia* (Macquart) (Diptera: Calliphoridae) larvae. Significantly higher ($p = .003$) levels of lead, barium, and antimony were found in larvae that fed on tissue beef that was shot rather than in the control. There was no difference within empty puparia casings (Roeterdink et al. 2004).

Challenges still exist for utilizing entomology as part of toxicological investigations. The greatest caveats to the field are the fact that absence of a drug in larvae does not mean absence in the human tissue (Wilson et al. 1993). Depending on the nature of the drug, the production of only qualitative, not quantitative results, show the lack of correlation between volume of toxins within maggots and cadavers (Tracqui et al. 2004). This last statement contradicts research done with *L. sericata*, *Calliphora vicina* (Robineau-Desvoidy) (Diptera: Calliphoridae), and *Protophormia terraenovae* (Robineau-Desvoidy) (Diptera: Calliphoridae) where correlations were found between levels of morphine in tissue and 3rd instars (Hedouin et al. 1999, Hedouin et al. 2001). Tropisms of drugs with respect to organs

explain different ratios between collected body samples and larvae, especially when multiple drugs are found within a body at once (Kintz et al. 1990c). This was the case in a 49-year-old male found dead by apparent suicide in which HPLC tested positive for multiple substances used in treating insomnia to seizures to depression. Triazolam (insomnia), oxazepam (insomnia), phenobarbital (seizures), alimemazine (sedative), and clomipramine (antidepressant) were all discovered within the maggots sampled and in completely different concentrations based on the tissues from which each larva was collected. However, as Tracqui et al. (2004) was quick to point out, most larvae are sampled off of the cadaver's surface where the drug may not be as present due to temperature, humidity, the nature of the drug, and pharmacokinetics with the body and larvae.

Furthermore, time of collection and life stage is important when utilizing entomotoxicology. Drug accumulation of four different drugs in *C. vicina* collected from three suicides was examined. In each case, the levels of trazodone, trimipramine, amitriptyline, and temazepam, decreased in the larvae after eight days of feeding and were untraceable by day 16 (Sadler et al. 1995). As well, further research with *C. vicina* recorded the effects of nordiazepam on larval growth rates and detection over the first five days of development. Extended observations showed that between the seventh and eighth days of sampling, nordiazepam was completely absent from the larvae. Furthermore, no difference in larval length or development time was noted (Pien et al. 2004).

Developmental Rates and PMI

There are two methods used for using arthropod evidence for determining the minimum-PMI (m-PMI) of an individual; succession, and arthropod development (Bygarski 2013). Succession can be defined as the biological principle where colonization of remains

occurs in a predictable sequence of waves represented by stages (Payne and King 1968). In each stage, the community structure of the arthropods present is compared to known succession patterns for the local area or habitat. This diversity level correlates with the stages of decomposition and the pattern of arthropod establishment (Eberhardt and Elliot 2008).

Estimates of the time of colonization (TOC) as related to a potential m-PMI can also be determined using development data for targeted arthropod species commonly associated with decomposing remains. Predominately, immature fly (Diptera) larvae are collected from deceased, or living humans in the case of myiasis, and used to estimate the TOC by determining the stage of development (e.g., instar) in conjunction with local temperatures in order to estimate an age. These stages are measured by tracking accumulated degree hours (ADH). ADH is a given amount of thermal energy needed to develop from one stage of an insect life cycle to the next based on one-hour increments (Simmons et al. 2010). Likewise, accumulated degree days (ADD) are the same measurement of thermal energy, but over a 24-h period. A predictable, temperature based developmental pattern allows this method to be utilized in nearly any situation where human, or other animal, remains are colonized by dipteran larvae for which published development data are available (Anderson 2000, Nabity et al. 2006, Niederegger 2010).

Prediction of the lower and upper thresholds is a questionable and evasive process. Indeed, testing a broad range of temperatures is essential in developing accurate rate models for insect development (Nabity et al. 2006). However, more often than not, these thresholds are either never reached or assumed the same for different populations of the same species and completely ignore fluctuation patterns. Ames and Turner (2003) reared populations of *C. vicina* and *Calliphora vomitoria* (Linnaeus) (Diptera: Calliphoridae) and exposed them to

varying cold periods where it was hypothesized development would cease upon reaching the lower threshold. In practice, however, showed that total ADH for both species decreased linearly with increasing temperature of the cold periods, suggesting continued development, rather than the absolute zero that is commonly assumed. These results gave rise to an alternative method in calculating growth rates where:

ADH = ((Dev time in hours – hours in the cold episode) x temperature after cold period))

+

(hours in the cold episode x temperature of the cold episode in Celsius)

Unfortunately, this formula assumes the temperature immediately after the cold period is constant. In an indoor case study, this may be the case, however, rarely do houses or apartments reach cold enough temperatures to hit assumed threshold levels. Furthermore, the rate in which the cold period reaches its lowest temperature, and the temperatures that determines when the period ends or begins is never discussed.

Perhaps the answers regarding threshold-levels lie in the temperature preference of larvae themselves. Studies by Byrd and Butler (1996, 1997) utilized a temperature gradient in which temperature changed from one area to the next, and observed the migration of larvae to their preferred zones. Interestingly enough, migrations changed depending on larval stage. *Cochliomyia macellaria* (Fabricius) (Diptera: Calliphoridae) migrated to 35°C in the first 24 h, 38°C after 48 h, and 31°C after 72 h before eventually migrating to pupate (Byrd and Butler 1996). In contrast, this preference does not necessarily equal optimum growth. It is possible that the temperature most comfortable for the maggot mass is not the optimum

temperature in which to maximize growth rate. Nor is there any speculation on a relationship to threshold temperatures. To date, there have been no papers published correlating preferred temperatures with growth rates and threshold levels.

There are a number of parameters associated with arthropod development that impact estimates of TOC. Data, such as larval weight, length, and life stage, are collected during development studies. These parameters are used to estimate the growth rate of larvae and can be impacted by a variety of biotic and abiotic factors.

Abiotic factors, such as temperature, light, radiation, soil, and humidity, impact the development of arthropods. *C. macellaria* from Texas, USA, displays an altered growth rate based on temperature where larvae reared at 28.2°C needed ≈31% and 21% fewer degree days to complete development than those reared at 20.8°C and 24.3°C, respectively (Boatright and Tomberlin 2010).

In another study, habitat in general seemed to effect colonization events between grass, short tree, tall tree, and urban environments. In total, urban environments were found to be colonized more frequently than tall tree areas by 56%, short tree zones by 49%, and grassland by 49% (George et al. 2013). However, this could be due to a larger base population in these areas rather than an oviposition preference.

Biotic factors that impact the development of fly larvae include, but are not limited to, the presence or absence of predators, and food source (tissue type and condition). The most widespread and rarely studied biotic factor affecting growth and survival of insects is the presence of parasites and parasitoids (Turchetto and Vanin 2004), however, this concept is relatively new and proper rearing of parasitoids for laboratory study can be difficult. Another, arguably equally important factor, coincidentally much easier to test, is the effect of

nutrient sources on the growth rate of insects. For example, the development rate for *L. sericata* immatures varied depending on if they were provided brain, lung, liver, kidney, heart, meat, or intestine (El-Moaty and Kheirallah 2013). Larvae raised on brain and lung grew ~35% faster than those raised on intestine. They also were 1.5 mm larger (>16%) and had a higher mean adult survivorship (85%) than the mean survivorship of those raised on liver, heart, or minced meat (≤60%) (El-Moaty and Kheirallah 2013). Similar results stemmed from an earlier paper with the same blowfly, but fed lung, liver, and heart from both bovine and porcine, liquefied and as a solid. Overall, larvae grew faster and larger on pork versus beef and on lung and heart versus liver (Clark et al. 2006). Likewise, *Lucilia cuprina* (Wiedemann) (Diptera: Calliphoridae) and *Calliphora augur* (Fabricius) (Diptera: Calliphoridae) developed slower and had smaller pupae on sheep liver versus muscle tissue and brain matter (Day and Wallman 2006). This suggests larval growth rate can be directly related to the type of tissue reared. On another note, in the case of *C. macellaria*, species origin of the tissue did not seem to make a difference in overall larval length, weight, or speed of development (Boatright and Tomberlin 2010).

Development data sets are available for many fly species from various regions of the world. However, a data set produced from one region might not be reliable when applied in a different region. This lack of reliability is due to the existence of population differences existing at the genetic level. Such variation could impact the responses of these populations to various environmental (abiotic and biotic) factors. While, some populations demonstrate a large amount of response variation (phenotypic plasticity) to a given stimulus (e.g., development in response to temperature) others might not. With regard to forensic

entomology, such information is of immense value as determining the appropriate data set for a given region is extremely important when attempting to accurately estimate the TOC.

Phenotypic plasticity has been examined for the development of *C. macellaria* from different regions in Texas, USA (Owings et al. 2014). Due to differences in climate within central, northeast, and southeast regions, genetic variances developed between the strains, and the resulting larvae displayed significantly different development times, mass, and survival rates, despite being the same species. Developmental and phenotypic plasticity were hypothesized as the causes for different growth rates between *L. sericata* collected from three different locations (Sacramento, CA, San Diego, CA, and Easton, MA) and tested at three different temperatures (16°C, 26°C, and 36°C). In the experiment, results showed a significant difference in all population growth rates with particular interest in the larvae from San Diego. Here the larval development time in a 16°C trial was 185 h as opposed to 207.5 h (Sacramento) and 210.5 h (Massachusetts). However, in the 36°C trial, the San Diego population took the longest to develop (74 h) while the other two shortened considerably resulting in 64.5 h with Sacramento and 66.5 h with Massachusetts populations. These results suggest climate impacts developmental progression at different temperatures even within the same fly species over generations. For this reason, having multiple data sets for the same species within differentiating climates and geography can be paramount to achieving an accurate TOC.

Finally, plasticity has been analyzed between field and laboratory studies as well. Development discrepancies were shown to exist between *L. sericata* raised with variations in laboratory conditions, and those reared under field conditions (Tarone and Foran 2006). Results showed *L. sericata* larvae given fresh meat daily developed in ≈ 200 h, while those

not given fresh meat daily took more than 250 h. Other significant differences were found with the introduction or absence of a paper towel for moisture control, destructive sampling versus non-destructive sampling, and the transfer of pupae to a substrate versus a practice of non-disturbance.

Despite obvious differences in development when exposed to the aforementioned factors, forensic entomology as a field has yet to truly standardize methods when conducting a developmental study. Primarily, this is due to an overall lack of agreement with standard operating procedures (SOP). As Tarone and Foran (2006) found, rearing methods can drastically change the way in which a subject can develop. However, it can be argued that a different SOP should be created and standardized per species and per case. With the possibility that, say, development of *C. macellaria* for an indoor case can be completely different from outdoors, as well as a case involving *L. sericata* indoors and outdoors, one could expect four entirely different ways in which to run a developmental study due to one factor alone!

In this effort, research should evolve around a common standard based entirely on actual casework. The scuttle fly, *Megaselia. scalaris*, for example, is typically associated with indoor cases, especially in cases involving well sealed rooms and structures (Greenberg and Wells 1998), and is among the most common genus within the family Phoridae (Disney 2008). Capable of occupying a large variety of niches through its cosmopolitan feeding behavior, *M. scalaris* has quickly become a topic in medicolegal, stored-product, and urban forensic entomology. Veterinary sciences, as well, are becoming aware of the species as the fly is seen in more and more veterinary casework (Vanin et al. 2013). Though there have been reports of their colonization outdoors, these are typically special in some form. In Bari,

Italy, a coffin buried roughly 30-40cm deep was found infested by the scuttle fly in question (Campobasso et al. 2004). In this case, there was no competition from other Dipteran species due to the sealing of the body beneath the ground while *M. scalaris* has been known to burrow and find corpses. In either case, they are known to be within the first wave of colonization both outdoors (Oliva 1997) and indoors (Oliva 2004).

This behavior allows *M. scalaris* to be an excellent candidate for creating a casework SOP for determining TOC. There are fewer biotic and abiotic factors within controlled environments whether that be in a house or in a buried coffin. Not only are temperature fluctuations less drastic, weather phenomena, predation, parasitism, humidity, and other factors are also reduced.

However, due to the lack of information regarding this species and its development within the United States, there are many questions to be asked. Owing most of its origins to biotic and abiotic factors combined with developmental and phenotypic plasticity, the question of whether or not tissue type, whether from different parts of the same species or of different species altogether, plays an important role in development continuously presents itself. Furthermore, as *M. scalaris* is renowned for being present during all stages of body decomposition, how does the condition of tissue affect growth rates within the species? As a species that has found its niche within more environmentally stable areas, have the margins of upper and lower thresholds become narrower? Considering phenotypic and developmental plasticity, are developmental rates changed from colonies collected in Europe different from those found in the southern USA?

All these questions and more should be considered when conducting a study with the intentions of use as entomological evidence. If one fails to include such factors in to their findings, results could be misleading.

Objectives and Hypothesis

Objective 1: Determine if tissue (*biceps femoris*) from two different vertebrate animals (porcine and bovine) significantly impacts growth rate of *M. scalaris.*

H_0: Tissue from porcine and bovine will have no significant effect on overall growth rate.

H_a: Tissue from porcine and bovine will have a significant effect on overall growth rate.

Objective 2: Determine if the growth rate of immature *M. scalaris* is impacted by temperatures.

H_0: *M. scalaris* larvae and pupae will show no difference in growth rate between 24ºC, 28ºC, and 32ºC when calculated by actual hours.

H_a: *M. scalaris* larvae and pupae will show significant growth rate differences between 24ºC, 28ºC, and 32ºC when calculated by actual hours.

CHAPTER II

RESEARCH, RESULTS, AND DISCUSSION

Introduction

Forensic entomology is the utilization of insects and other arthropods as evidence in both civil and criminal investigations (Smith 1986), as well as within non-criminal investigations of death or myiasis in general. The broad scope of this field can be broken down into three subdivisions, which are urban, stored-product, and medicolegal entomology (Archer 2007). Medicolegal entomology relates to necrophagous arthropods colonizing and feeding on living and necrotic human and animal tissue. Insects found on vertebrate remains may be utilized to estimate the time of colonization (TOC) which can then be used to estimate the minimum-PMI (Amendt et al. 2007).

Entomologists can be asked to analyze insects collected from living people and animals. Confirming myiasis, the infestation and subsequent feeding upon living tissue, organs, or bodily fluids of a vertebrate host by dipteran larvae, can be used as evidence in cases of suspected neglect or abuse (Anderson and Huitson 2004). Myiasis typically occurs in elderly or very young people, who are unable to care for themselves, as well as in pets and livestock with open wounds or fecal matter present on their bodies for an extended period of time (Anderson and Huitson 2004). The forensic entomologist then uses the same techniques to estimate the TOC related to the infestation of the wound and timing related to the abuse or neglect.

Forensic entomologists depend on growth data from laboratory studies to estimate the TOC for blow flies (Diptera: Calliphoridae) collected from decedents (Tarone and Foran

2006) as well as the living (Anderson and Huitson 2004). Although similar development times have been documented in pre-existing data sets for a variety of blow fly species, some variations can be found within species (Tarone and Foran 2006, Gallagher et al. 2010). Variation could be due to a number of factors, such as experimental design, environment and genetic variation. Tarone and Foran (2006) determined that environmental conditions greatly influenced the development of the blow fly *Lucilia sericata* (Meigen) (Diptera: Calliphoridae). Their findings demonstrate the need for multiple development data sets for each species of blow fly across geographic regions, as well as for a standardization of laboratory rearing techniques.

Most development data utilized by forensic entomologists are obtained from studies examining the growth of larvae fed beef liver (Clark et al. 2006). However, it has been demonstrated that the host species on which the larvae feed may significantly alter larval growth rate. For example, development of *L. sericata* fed lung, liver and heart, from both cows and swine were compared (Clark et al. 2006). It was determined that larvae grew significantly faster on swine than on cow. Furthermore, development varied when reared on lung and heart compared with liver of the same animal. For forensic entomologists, such results emphasize the need for development data sets across tissue types as well as across species. The importance of recording the location from which blow fly larvae are removed from a corpse at a body recovery scene was also demonstrated (Clark et al. 2006). Such information can allow for refined estimates of the period of insect activity.

Globally, *Megaselia. scalaris* (Loew) (Diptera: Phoridae) is considered among the most common of the Phoridae family (Disney 2008). This is due to their woldwide

distribution and ability to adapt to a large variety of environments. Hopefully this research will inspire other similar studies with multiple Diptera of forensic importance.

Though *M. scalaris* is widely encountered in forensic entomology investigations, little is actually known about the biology of this species. Flies from the family Phoridae are commonly known as hunchback flies, scuttle flies, or coffin flies due in part to their morphology, behavior, and food source respectively. Cosmopolitan by nature, *M. scalaris* can be a scavenger, parasite (Miranda et al. 2011), or detritivore (Koch et al. 2012). Utilizing diverse habitats from tropical rain forests to heavily urbanized areas, the wide distribution of *M. scalaris* makes it both a residential pest, and an excellent candidate for forensic entomology casework (Disney 2012). This species has been encountered on human remains located indoors in a sealed building where the small size of the phorids allows them easier access to a corpse, than the larger blow fly species (Reibe and Madea 2010).

Though developmental data exist for *M. scalaris*, these experiments are based on populations either from other countries such as Mexico (Miranda et al. 2011) and Malaysia (Zuha and Omar 2014), or from old publications in North America likely in long-term lab colonies (Greenberg and Wells 1998, Prawirodisastro and Benjamin 1979, Trumble and Pienkowski 1979). Likewise, there are concerns that genetic differences between populations may result in developmental differences and caution should be exercised when applying these data to cases outside these countries and climates. Such differences have already been recorded with oriental latrine flies (Hu et al. 2010) where *Chrysomya megacephala* (Fabricius) (Diptera: Calliphoridae) populations from different regions in China along the same latitudinal gradient within different environments demonstrated slowed or increased

overall development times. Furthermore, there has been no research, as of the writing of this paper, comparing the relationship between both temperature and species specific tissue effects on development of *M. scalaris*.

In the field of forensic entomology, accurate developmental data are imperative when analyzing arthropod samples as evidence in a death investigation. Reliability of an expert witness and his or her findings are paramount to any case. So much so that Congress directed the National Academy of Sciences to undertake a study that lead to what is commonly known as the 2009 National Academy of Science (NAS) Report. A committee was created to "identify the needs of the forensic science community." Titled Strengthening Forensic Science in the United States the report focused on solidifying the importance of standards, certifications, accreditation, and funding in all fields of forensic science.

Currently, no data sets are available on the development of *M. scalaris* in Texas, USA. Due to the importance of *M. scalaris* in forensic entomology, specifically for indoor cases, more accurate developmental data must be produced to satisfy the challenges set forth in the NAS report. This issue is specifically relevant to the Harris County Institute of Forensic Sciences where nearly 50% of cases during 2013-2014 involved *M. scalaris* as a primary colonizer of human remains (M. Sanford, *personal communication*). The current data are too incomplete to provide modern day forensic entomologists with the most accurate determinations possible in casework. The objective of this study is to fill in the gaps caused by phenotypic and developmental variation with biotic or abiotic factors, and aid in the standardization of methods used by forensic entomologists in the United States.

Materials and Methods

Adult Fly Colony

The *M. scalaris* colony used in this experiment originated from larvae collected off human female remains from a case assigned to the Harris County Institute of Forensic Sciences on October 9th 2014. Larvae were provided 50 g of fresh bovine liver within their transportation container and allowed to pupate.

Adult flies were housed in a nylon mesh 30 x 30 x 30 cm BioQuip™ (Rancho Dominguez, CA, USA) arthropod-rearing cage and provided a 50:50 sugar: powdered milk mixture and fresh water (Byrd and Butler 1996). Temperature fluctuated between 24ºC and 28ºC with an alternating 12:12 light and dark cycle. Approximately 50 g of bovine liver in a small petri dish was provided to the colony for three hours as an oviposition substrate. Previous observations showed the oviposition rate was much higher when substrate was placed on a petri dish rather than an 88.7 ml plastic cup. This is likely do to less crowding and more surface area in which to lay eggs. As the behavior of *M. scalaris* prefers surface movement over flying, and laying eggs singly in different areas of a substrate rather than in batches, this wide area in which to move combined with a hard surface in which to let blood and decomposition fluids to pool, provides an excellent stimulant for egg laying production. Resulting eggs and associated liver were placed in 236.5 ml, wide mouth Kerr™ mason jar (Hearthmark, Daleville, IN) for rearing in the same room under the same conditions previously mentioned. Emergent adults were collected daily and placed in a cage similar to the one previously described in groups by generation.

Source

The *biceps femoris* muscle from remains of two bovines, *Bos taurus* (Linnaeus), and two porcines, *Sus scrofa* (Linnaeus), were utilized in this development study. All tissue was acquired from a local butcher shop (Readfield Meats and Deli, College Station, TX, USA). All tissue utilized were free of barbiturates or other materials used for euthanization. Each piece of tissue was cut in varying shapes, thickness, and pieces to weigh in at 50 g per Ziploc bag, labeled by their respective tissue source and date, and kept within a freezer at -20°C. All protocols were approved by the Texas A&M Animal Care and Use Committee (IACUC), as well as the Animal Welfare Assurance Program, Texas A&M University, College Station, TX.

Life-History Traits

Methods were based on those of Boatright and Tomberlin (2010). Tissue samples were removed from the freezer and allowed to thaw and reach room temperature prior to use. Approximately 50 g of either bovine or porcine tissue was placed in a 236.5 ml wide mouth Kerr™ mason jar filled a quarter way with sand (Quikrete Premium Play Sand, Atlanta, GA) to prevent pooling of decomposition fluids possibly killing the larvae. Six jars were prepared for each tissue type, 12 in total per trial. Each jar was inoculated with 400 eggs from the colony's F7-F9 generation. Each jar was then covered with a paper towel and secured with a metal ring (band). This method allowed airflow and prevented any emerging adults from escaping the enclosure. The jars were then randomly assigned, by tissue, to one of three 136LLVL Percival® upright incubators set at 24.0°C ± 1.0°C, 28.0°C ± 1.0°C, or 32.0°C ±

1.0°C. Light cycle and humidity in each incubator was set to 12:12 (L:D), and 55% ± 5% RH respectively. Three trials were conducted so that each incubator was observed with each temperature. During the study, four Onset® HOBO U12-006 data loggers (Onset Co., Pocasset, MA) were placed within four separate parts of the incubator to test for variability between shelf height (top shelves versus bottom shelves) and shelf sides (left side of the shelf versus the right). Furthermore, each unit was rotated within the sections of the incubators to measure against differences between the data loggers. Each section measured at ± 1ºC and ± 1% shift in relative humidity (RH), with jars closer to the fans above having the lowest humidity and temperature, while those near the bottom, where the water tray is located, having the highest RH and temperature. As these numbers fell within the estimated errors, location within the incubators was assumed to have no effect.

Development time was recorded between egg to larval eclosion, hatch to pupation, pupation to adult emergence, and overall hatch to adult emergence. Eggs in each sample were checked hourly for hatch. Following eclosion, observations were conducted every 12 h in which three larvae were sub-sampled from separate areas within the tissue. Each was placed within a Brew & Save reusable filter cup (Eko Brands, LLC. Mill Creek, WA) and parboiled in water for no more than 15 s. This method allowed room for the full expansion of the sub-sampled larvae while preventing accidental escape or loss of the, at times, extremely small (less than 1.5 mm) specimens. Each was measured for length in millimeters using a Meiji Techno® EMZ-8TR microscope (Meiji Techno America, Santa Clara, CA). Weight was not measured due to the AdventurerPro AV64 weighing scale (Ohaus, Pine Brook, NJ) not being able to accurately read the weight of much of the feeding larvae stages of *M. scalaris,* or

discern the difference between larvae and water evaporation. As larvae are typically moist, any dry weight measurements would be inaccurate.

Due to brittleness and overall sensitivity, phorid pupae were left undisturbed within their respective jars and were not placed in separate containers. Jars were checked every 12 h for adult emergence so as not to completely bury or destroy the pupae within. Each jar was monitored every 12 h for adult emergence. Number of adults emerging during each observation were collected, sexed, and tabulated.

Statistical Analysis

All statistical analyses were performed using JMP statistical software (SAS Institute Inc., Cary, NC) and Microsoft Office Excel version 2007 (Microsoft Corp., Redmond, WA). The analysis called for a repeated measures split plot design with tissue type as the whole plot treatment, temperature as the split plot treatment, and time as the repeated measures factor, and separate incubators as random blocks. Length and rate of development were recorded in both natural hours and ADH. Tukey's honestly significant difference test (Tukey's HSD) was utilized to separate least square means following a significant F test ($P < 0.05$).

Preliminary Validation of Development Data

A preliminary validation study was conducted utilizing the minimum and maximum lengths and ADH ranges of *M. scalaris* recorded in this developmental study. Validations were run in four different locations throughout two buildings of the Forensic Laboratory of Investigative Entomological Sciences (F.L.I.E.S.) Facility at Texas A&M University, College Station, TX, USA.

Utilizing the same methods as conducted within the development study, approximately 50 g of either bovine or porcine tissue was placed in a 236.5 ml wide mouth Kerr™ mason jar filled a quarter way with sand (Quikrete Premium Play Sand, Atlanta, GA). All eggs were pulled from the colony and portioned with two jars containing bovine muscle and 100 *M. scalaris* eggs each, no more than 3-h-old, as well as two jars containing porcine muscle also inoculated with 100 eggs each of the same age, and placed in four indoor locations. One jar containing beef with eggs and one containing pork with eggs was placed in two separate attics in buildings 1043 (Building/Attic A) and 1050 (Building/Attic B), 29.87m (L) x 12.19m (W) x 3.15m (H), and 18.29m x 12.19m x 3.15m, respectfully. Likewise, another placement of pork was located in a shower (0.64m x 0.71m x 2.49 m), and the final jar of beef was found within a closet (1.14m x 2.06m x 2.06m). All jars were immediately placed in their respective areas immediately after being collected from the colony, by an outside laboratory member at a time determined by the associate only, which was later revealed as between 1900 and 2000 May 11, 2015. Jars were pulled at times determined by the same outside laboratory member: two on 13 May at 1000 and 1900 (Shower room and Attic B attic, respectfully), and two on 14 May at 0900 and 1500 (Closet and Attic A, respectfully). These times were selected by the associate in an effort to sample all larvae before pupation, but across multiple instars. A range for time of colonization was determined upon collection of each sample using recorded ADH in combination with maximum, minimum, and average larval lengths. Temperatures in each site were closest to the lowest temperature (24ºC) utilized in the study; therefore, ADH and length was calculated based on development data collected for that temperature.

Results

Life-History Traits

There was not a significant interaction effect of temperature by tissue on larval length ($F_{2, 10.53} = 0.0852$, $P = 0.9189$) or development time ($F_{2, 5.564} = 0.0884$, $P = 0.9167$). Degree hour (DH) data for *M. scalaris* development when reared on bovine and porcine tissue are shown in Table 4 using a base temperature of 10°C. Immature development with regard to time (Table 4) and length (Figure 1) are presented.

In actual hours, time needed for egg hatch was significantly ($F_{2, 23.24} = 157.0285$, $P < 0.0001$) impacted by temperature. From 24ºC to 28ºC an average difference across all trials was observed at 4.7 h, 1.6 h between 28ºC to 32ºC, and 6.3 h between 24ºC and 32ºC. Likewise development from hatch to pupation, as well as pupae to adult, was significantly (hatch to pupa: $F_2 = 173.2503$, $P < 0.0001$; pupa to adult: $F_{2, 6.144} = 976.9159$, $P < 0.0001$) impacted by temperature. An average difference of 20 h was observed between larvae exposed to 24ºC and 28ºC. Development of pupae to adult when reared at the same temperature was separated by as much as 100 h. Larval development between 28ºC and 32ºC was separated by 8 h, but only 12.3 h from pupation to adult. Lastly, an average 36 h difference was recorded between hatch to pupation for larvae reared in 24ºC and 32ºC, and spent 112.3 h longer in pupation before adult emergence.

Larval length was also significantly ($F_{2, 10.53} = 10.6060$, $P < 0.0051$) impacted by temperature. Throughout the study, however, tissue did not significantly ($F_{1, 5.41} = 0.3486$, $P = 0.5787$) affect larval length or larval development ($F_{1, 4.116} = 0.0058$, $P = 0.9430$).

Validation of Developmental Data

Validation results are shown in Table 6. Relative humidity was significantly greater (F_3 = 3876.788, P < 0.0001) than the measured RH during the control study in all areas except for the closet located in Building A. Relative humidity averaged 82% in Attic A, 75% in Attic B, and 74% in Building A's shower room. Furthermore, temperatures across all field sites averaged 22.0ºC ± 0.4ºC. As well, the temperature in Attic A fluctuated between 21.0ºC ± 0.5ºC and 31.0ºC ± 0.5ºC.

An entire range of matching minimum, maximum, and mean TOC was cross-referenced with larval lengths. Maximum length was the closest in all observations to the actual TOC. Within Attic A, all measures resulted in estimates less than the actual TOC. When using the minimum length of the larvae sampled, the estimate was 58% (39h) less than the actual TOC, but when using the maximum length, the estimate was 1.5% (1h) less than the actual TOC and 30% (20h) less when utilizing the average length. TOC calculations for specimens from Attic B were underestimated by 40.4% (19h), 10.6% (5h), and 25.5% (12h) (minimum, maximum, and mean, respectfully). For the shower room, the minimum larval length was underestimated by 26% (10h). However, using maximum larval length the actual TOC overestimated by 10.5% (4h) and, average larval length underestimated the actual TOC by 8% (3h). Lastly, the closet space produced similar over-under estimations as the shower room in that minimum underestimated TOC by 54% (33h), maximum overestimated the actual TOC by 10% (5h), and the average as well underestimated actual TOC by 23% (14h).

Discussion

To the date of this study, there is no other published research examining the impact of temperature and tissue type on the development of *M. scalaris*. Furthermore, this study provided a preliminary assessment of the accuracy of these data for determining the time of colonization. Such data are imperative for estimating a TOC in a case study or investigation.

Conditions used in development studies are rarely the same as those experienced during actual casework. Often, many abiotic and biotic factors impact development patterns of arthropods associated with decomposing human remains, much more than those in controlled laboratory studies. Thus, a validation study is useful for determining the accuracy of the lab-generated data when applying them in casework. Furthermore, these validations should be expanded beyond the localized area of the study in order to assess regional variability.

The most recent developmental study on *M. scalaris* was conducted in Malaysia (Zuha and Omar 2014). Overall, development times between egg and adult emergence for *M. scalaris* in this study were faster in actual accumulated hours than those found in Zuha and Omar (2014). Ranges in development were from 381.125 h at 24°C in this study, to 417.5 h at 23°C and 304 h at 25°C in Zuha and Omar (2014). Likewise, average hours taken for full development at 32°C was 249.2 h in Zuha and Omar (2014), but only 234.7 h in this study. These differences in development in *M. scalaris* across studies could be due to a number of previously discussed factors.

One of the most important, and often overlooked factors impacting arthropod development is food quality. In an earlier study with *M. scalaris*, immature development

rates were enhanced when fed raw beef liver rather than an artificial diet of liver agar. As much as an 8 h difference was determined when reared on these two diets at 27ºC, 10 h for 30ºC, and 15.4 h in 33ºC (Zuha et al. 2012). Within other forensically important Diptera, the development rate for *L. sericata* immatures varied depending on if they were provided brain, lung, liver, kidney, heart, meat, or intestine (El-Moaty and Kheirallah 2013). Larvae raised on brain and lung grew ~35% faster than those raised on intestine. They also were 1.5 mm larger (>16%) and had a higher mean adult survivorship (85%) than the mean survivorship of those raised on liver, heart, or minced meat (≤60%) (El-Moaty and Kheirallah 2013). Similar results stemmed from an earlier paper with the same blowfly, but fed lung, liver, and heart from both bovine and porcine, liquefied and as a solid. Overall, larvae grew faster and larger on pork versus beef and on lung and heart versus liver (Clark et al. 2006). Likewise, *Lucilia cuprina* (Wiedemann) (Diptera: Calliphoridae) and *Calliphora augur* (Fabricius) (Diptera: Calliphoridae) took two days longer to develop on sheep liver versus muscle tissue and brain matter (Day and Wallman 2006).

Population differences due to location of study have been shown to be a factor impacting development. Phenotypic plasticity has been examined for the development of *C. macellaria* from different regions in Texas, USA (Owings et al. 2014). Due to differences in climate within central, northeast, and southeast regions, genetic variances developed between the strains, and the resulting larvae displayed significantly different development times, mass, and survival rates, despite being the same species. Furthermore, mean development time by cool versus warm environments, and year of observation made an impact as well. The population from Longview, TX displayed shorter developmental times by up to 17 h

when compared to those from College Station, TX and San Marcos, TX during the cool seasons, and up to 18 h during the warmer months. However, the following year had Longview populations with an increased time of development by 9 h during cool months and 16 h when warm. Similar results were determined for different growth rates between *L. sericata* collected from three different locations (Sacramento, CA, San Diego, CA, and Easton, MA) and tested at three different temperatures (16°C, 26°C, and 36°C) (Gallagher et al. 2010). They determined a significant difference in growth rates across all populations especially for those from San Diego, CA where the larval development time at 16°C was 185 h as opposed to 207.5 h for the Sacramento, CA population and 210.5 h (Massachusetts, USA). However, when raised at 36°C, the San Diego, CA population took the longest to develop (74 h) while the other two needed 64.5 h (Sacramento, CA) and 66.5 h (Easton, MA). These results suggest climate impacts developmental progression at different temperatures even within the same fly species over generations. For this reason, having multiple data sets for the same species within differentiating climates and geography is critical to achieving an accurate TOC.

Variation in the methods employed across the studies has also been shown to be a source of variation in development of forensically relevant arthropods. Developmental plasticity has been analyzed between field and laboratory studies. Developmental discrepancies were shown to exist between *L. sericata* raised under variable laboratory conditions, and those reared in field conditions (Tarone and Foran 2006).

Although the validation study was preliminary, some appreciation for the accuracy when applying the generated development data for *M. scalaris* to case studies was

determined. It is important to note that despite the abiotic differences within each validation environment and the study itself, all four validation means were underestimated, but accurate within 20 h, with the largest span being that of the fluctuating temperature combined with the much greater RH levels in Attic A. The lowest difference of 3 h was in the shower area of Attic A, despite having similarly high humidity measured in the area, further suggesting RH starts to effect development times and lengths during the later stages of larval development over the early stages immediately following egg hatch.

Although an informative and useful study, there were limitations. Primarily, the lack of weight measurements and the inability to determine instar of the developing larvae are critical weak points in this study. In the case of *Chrysomya megacephala*, instar data was more effective than size for estimating the age of young larvae, however after 72 – 120 h, neither size nor instar could give an accurate age determination (Wells and Kurahashi 1994). Furthermore, due to different kinds of preservative fluid, body length may be influenced in preserved larvae (Tantawi and Greenberg 1993). Due to the small size and mass of *M. scalaris* larvae, instar determination and weight is extremely difficult to measure accurately before the third instar, and often require a scanning electron microscopy (SEM) (Boonchu et al. 2004) and an extremely accurate weighing scale to account for both limitations. As well, even though the colony population originated from a local case, multiple populations or an addition of local, wild populations would be useful. Periodically introducing wild adults from different locations within the immediate area could not only eliminate generation effects, but laboratory colony effects and increase genetic diversity. Finally, as previously stated, the validation study was preliminary. Though there were four samples taken from four different areas, the study lacked replication. Multiple replicates and trials should not be limited to lab

work alone, and should be included when planning a validation study to test data sets when at all possible. A final note on validations considers the use of human and companion animal cadavers as the best test of accuracy.

Development studies serve as a primary method for estimating the TOC in forensic case studies, criminal or otherwise. Accurate results and understanding of all data sets for each dipteran species in question throughout a variety of separate factors is critical to obtaining the best possible estimation for TOC. Laboratory trials should attempt to replicate these effects as closely as possible either in the control itself, or by applying the data to a field study incorporating such factors. Using data collected from both temperature and tissue type, one could effectively create a more useful data plot to estimate the TOC in any case, criminal or otherwise, when compared to data of either tissue or temperature alone. Besides tissue comparison between different vertebrates, one should consider where larvae are sampled in comparison to the body, as some insects, such as *C. vicina*, develop differently on various organs and tissues than others due to nutrition content (Kaneshrajah and Turner 2004). Hopefully this research will inspire similar study variations involving many biotic and abiotic factors and multiple Diptera of forensic importance.

CHAPTER III

SUMMARY OF LIMITATIONS AND FUTURE RESEARCH

As previously mentioned, there were a few limitations to the study that should be considered. For example, weight was not recorded due to the scales not accurately measuring the extremely miniscule mass of the larvae. Due to this, tissue effect on larval weight was not measured.

Based on other limiting factors and observations found during the validation, any further developmental studies should focus on temperature and/or humidity fluctuation, humidity, lower and higher temperatures, tissue effects on weight, tissue effects of human tissue, and differences in growth rates on different organs or other tissues. Though no repetition effect was found, I do wonder about the varying methods utilized in various developmental studies and how they impact *M. scalaris* in particular.

On this note, feeding, disturbance, and wandering behavior of dipteran larvae in question should be a factor when measuring growth rates. Throughout my observations, I had larvae of all stages burrowing deep from the tissue source. As well, some larvae preferred to pupate in their own tunnel system, or on top of the substrate itself, while others followed the much more popularized behavior of climbing to pupate on the jar walls, as often seen within indoor cases (M. Sanford, *personal communicatio*n). Could the availability of burrowing substrate versus the forced migration to a hardened surface in order to escape the tissue source have an effect on overall development? If so, this could mean extra care and observation should be made on the forensic entomologist's part to determine the migration behaviors of the specimens collected.

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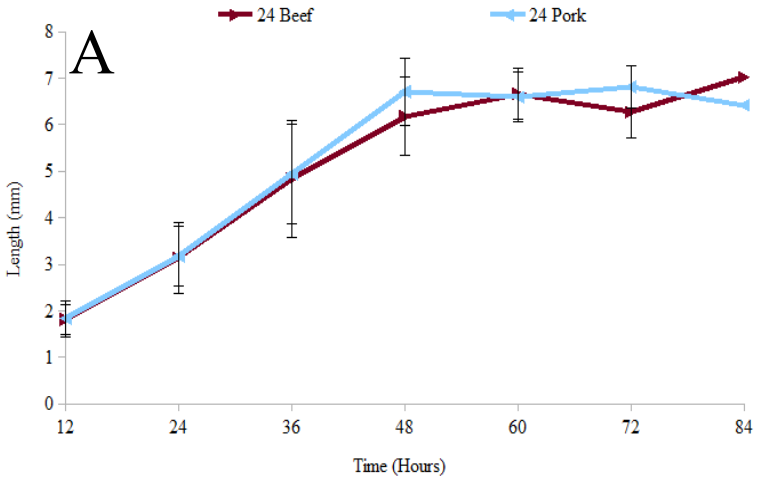
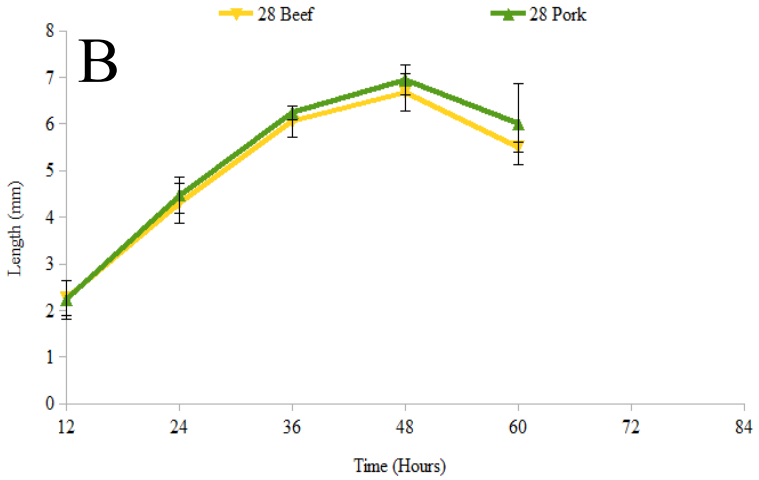
APPENDIX A
FIGURES





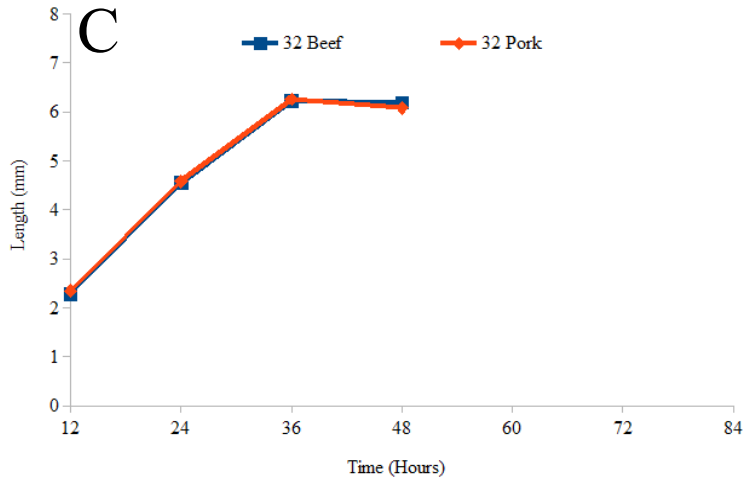
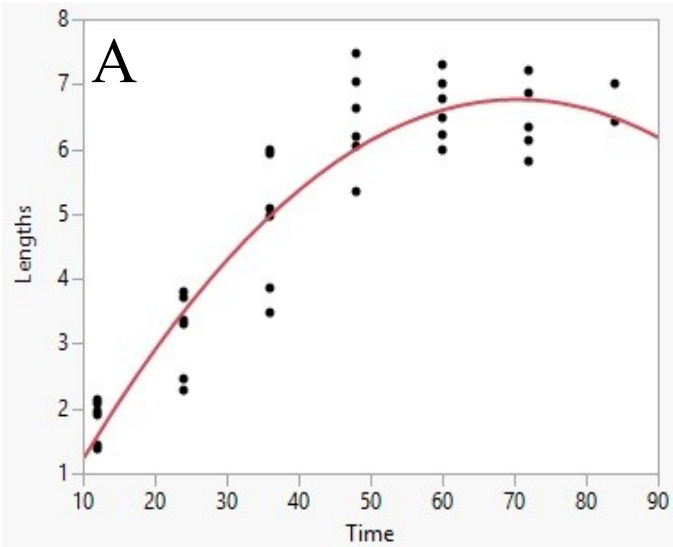
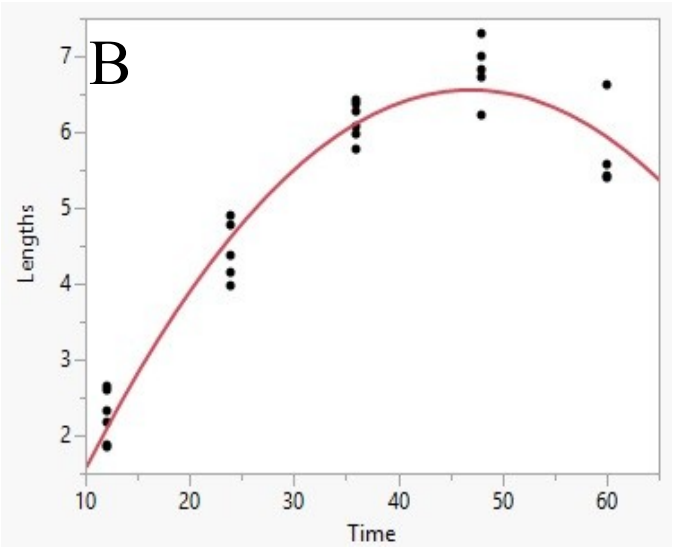


Figure 1. Length Over Time. Mean length (mm) ± SD of sampled *Megaselia scalaris* larvae over time (h) in 12 h intervals between temperatures 24ºC (A), 28ºC (B), and 32ºC (C) all ± 1.5°C and with two different tissues (beef and pork). Larvae were raised in 55% ± 1.0% RH in a 12:12 L:D.





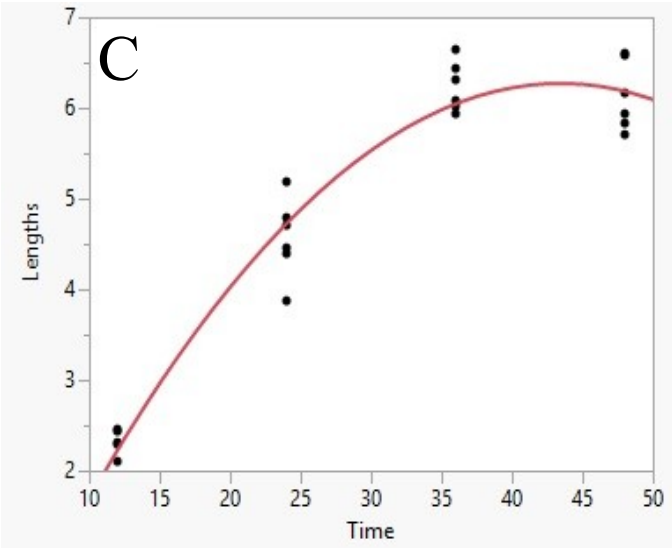


Figure 2. Quadratic Length Over Time. Mean length (mm) ± SD of sampled *Megaselia scalaris* larvae over time (h) in 12 h intervals between temperatures 24ºC (A), 28ºC (B), and 32ºC © all ± 1.5°C with a quadratic polynomial fit. Larvae were raised in 55% ± 1.0% RH in a 12:12 L:D.

APPENDIX B
TABLES

Temperature on Tissue

Time (Hours)	32°C Beef	32°C Pork	28°C Beef	28°C Pork	24°C Beef	24°C Pork
12	2.29	2.35	2.26	2.23	1.81	1.83
24	4.55	4.58	4.30	4.47	3.14	3.18
36	6.23	6.26	6.05	6.24	4.83	4.94
48	6.19	6.08	6.68	6.95	6.19	6.71
60			5.50	6.01	6.67	6.59
72					6.27	6.81
84					7.02	6.42

Table 3. Numerical values for Figure 1. Mean length (mm) ± SD of sampled Megaselia scalaris larvae over time (h) in 12 h intervals between three different temperatures (32ºC, 28ºC, 24ºC all ± 1.5°C) and two different tissues (beef and pork). Larvae were raised in 55% ± 1% RH in a 12:12 L:D.

Table 4. Minimum ADH for Development. Minimum degree hours and accumulated degree hours for development stages of *Megaselia scalaris* between three different temperatures (32ºC, 28ºC, 24ºC all ± 1.5°C) and two different tissues (beef and pork). Larvae were raised in 55% ± 1.0% RH in a 12:12 L:D.

Temperature (ºC)	Tissue	Egg to Hatch ± SE Degree Hours (DH)	Hatch to Pupation ± SE (DH / ADH)	Pupation to Adult ± SE (DH / ADH)
32 ± 1.5	Bovine	251.2 ± 8.4	1320.0 ± 33.9 / 1571.2	3864.0 ± 43.4 / 5353.8
	Porcine	253.0 ± 9.2	1320.0 ± 37.1 / 1573.0	3843.0 ± 43.4 / 5336.3
28 ± 1.5	Bovine	246.0 ± 8.4	1224.0 ± 33.9 / 1470.0	3150.0 ± 43.4 / 4620.0
	Porcine	246.0 ± 8.4	1224.0 ± 33.9 / 1470.0	3159.0 ± 43.4 / 4629.0
24 ± 1.5	Bovine	257.8 ± 8.4	1232.0 ± 33.9 / 1489.8	3864.0 ± 43.4 / 5353.8
	Porcine	261.3 ± 8.4	1232.0 ± 33.9 / 1493.3	3843.0 ± 43.4 / 5336.3

Table 5. Percent Difference of Development Time. Percent difference of *Megaselia scalaris* mean time of development from egg to adult emergence of *M. scalaris* between three different temperatures (32ºC, 28ºC, 24ºC all ± 1.5°C) and two different tissues (beef and pork). Larvae were raised in 55% ± 1.0% RH in a 12:12 L:D.

		Pork			**Beef**	
	32°C ± 1.5°C	**28°C ± 1.5°C**	**24°C ± 1.5°C**	**32°C ± 1.5°C**	**28°C ± 1.5°C**	**24°C ± 1.5°C**
32°C ± 1.5°C Pork	0.0%	10.5%	3.4%	-	-	-
28°C ± 1.5°C Pork	10.5%	0.0%	13.5%	-	-	-
24°C ± 1.5°C Pork	3.4%	13.5%	0.0%	-	-	-
32°C ± 1.5°C Beef	-	-	-	0.0%	8.5%	38.4%
28°C ± 1.5°C Beef	-	-	-	8.5%	0.0%	32.7%
24°C ± 1.5°C Beef	-	-	-	38.4%	32.7%	0.0%

Table 6. Validation Study Estimates. Development of *Megaselia. scalaris* within four locations in two different buildings of the Texas A&M F.L.I.E.S Facility to the minimum, maximum and average estimated times based on sampled larvae length from the lab study at 24ºC. Percentages shown are differences between the estimate and the actual time in field (h).

Location	Number Larvae Sampled	Time in Field (AAH[1]/ADH[2])	Minimum Estimate (AAH/ADH)	Maximum Estimate (AAH/ADH)	Mean Estimate (AAH/ADH)
Building B: Attic B	19	47 / 573	28 (-40%) / 335	42 (-11%) / 499	35 (-26%) / 417
Building A: Attic A	49	67 / 862	28 (-58%) / 386	66 (-1%) / 848	47 (-30%) / 617
Building A: Closet	45	61 / 785	28 (-54%) / 366	66 (8%) / 849	47 (-23%) / 607
Building A: Shower	7	38 / 516	28 (-26%) / 377	42 (11%) / 574	35 (-8%) / 475

[1]accumulated actual hours; [2]accumulated degree hours