ABSTRACT

CAMMACK, JONATHAN ALAN. Advancing Forensic Entomology in Indoor Environments: A Comprehensive Study From Colonization to Dispersal. (Under the direction of D. Wes Watson and K. Lane Kreitlow).

The decomposition of pig carrion in concealed and open environments was studied during the spring, summer, and fall in Raleigh, North Carolina. Concealment ranged from minimal to well concealed, in simulated attics, and significantly affected the insect community present. Although numerous species colonized remains indoors, the beetles *Necrobia rufipes* and *Dermestes maculatus* were the only species indicative of indoor remains. In contrast, numerous flies, beetles, and an ant, were indicative of remains outdoors. Season also significantly influenced the blow flies colonizing remains. A positive relationship between the degree of concealment and delay of colonization was seen during all seasons; concealment delayed colonization by 35-768 hours. *Lucilia illustris* was indicative of spring, *Cochliomyia macellaria* and *Chrysomya rufifacies* indicative of summer, and *Calliphora vicina* and *Calliphora vomitoria* indicative of fall. These differences between the insect communities across seasons and concealment treatments, and the effects of concealment on colonization indicate that such information should be considered when analyzing entomological evidence.

The concealment of remains often results in delayed discovery. Traditional methods of estimating the minimum PMI (mPMI) rely on the blow fly larvae collected from remains. However, if sufficient time has passed, these insects may have completed development and be present as adults, which are often ignored because of a presumed little relevance. In a laboratory study, pteridine fluorescence was quantified in the eyes of three blow fly species that colonize remains indoors: *Chrysomya megacephala*, *Cochliomyia macellaria*, and

Phormia regina. Age could be predicted with a high degree of accuracy (mean r^2 ± SE: 0.90 ± 0.01) for all species*sex*rearing temperature combinations except for *P. regina* at 5.4°C. The high r^2 indicates that this is a valid method for aging blow flies at temperatures above 15°C, and will increase the precision of mPMI estimates for concealed remains.

Accurate temperature data from a body-recovery scene are required for the analysis of entomological evidence. Forensic entomologists typically use regression analyses to predict on-site temperatures during the time prior to the collection of entomological evidence from remains. In this study, we investigated how the amount of time elapsed between the discovery of remains and temperature data collection, and how the duration of data collection, affects the accuracy of these regression analyses. As the time between discovery and data collection increases, longer durations of data collection are necessary to produce accurate models.

Due to their sarcosaprophagous nature, blow fly larvae are reared on an animal tissue diet. However, storage requirements and the associated odors can make these diets undesirable for laboratory use. In this study, the utility of three oligidic diets (rehydrated dry fish, dry dog, and dry cat food) for rearing *Lucilia sericata* was investigated. Fish food was not a suitable, but dog and cat food were. Pupae were significantly smaller when reared on these diets in comparison with beef liver or ground pork. However, diet had no effect on development rate or adult longevity, suggesting that dog and cat food are suitable for maintaining colonies. A diet of cat food was used exclusively to rear four additional blow fly species over approximately 3 years. The ease of storage and preparation of this diet, along with a reduced odor make it advantageous for laboratory rearing of blow flies.

The final chapter presents a review of insect colonization and laboratory rearing literature. Data are presented to show that numerous variables associated insect rearing can alter the rate at which an insect develops, which can confound the conclusions drawn from development studies. The implications for forensic entomology are discussed, and recommendations made to advance research on the development of forensically-important insects.

Advancing Forensic Entomology in Indoor Environments: A Comprehensive Study From Colonization to Dispersal

by
Jonathan Alan Cammack

A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Entomology

Raleigh, North Carolina

2013

APPROVED BY:

_____	_____
D. Wes Watson Committee Co-Chair	K. Lane Kreitlow Committee Co-Chair
_____	_____
Allen C. Cohen	R. Michael Roe

DEDICATION

To my 8th grade science teacher, Mrs. Patsy Knotts; thank you for finding the entomologist in me.

BIOGRAPHY

Jonathan Alan Cammack was born in Carrollton, Texas to Mark and Janet Cammack. He attended Texas A&M University, and graduated with a Bachelor of Science in Entomology, with a minor in Anthropology, in 2007. He then attended Clemson University, and graduated with a Master of Science in Entomology in 2009. Upon completion of his M.S. degree, he enrolled in the Department of Entomology at North Carolina State University to pursue a Ph.D.

ACKNOWLEDGMENTS

Thanks to my advisor, Wes Watson, for taking a chance, and allowing me to work on whatever I wanted. Thank you also for "letting" me work on all the other projects in the lab. Except for the so-called "mutiny" of 2011, learning about chickens, turkeys, pigs, and dairy cows was truly a fun and great experience. Thanks to my co-advisor Lane Kreitlow, and committee members Allen Cohen, and Mike Roe for their guidance and assistance in making my research successful. Thanks to Steve Denning for everything; none of this work would have been possible without his ingenuity and constant harassment, and his compulsion to make things look nice for pictures. Thanks to Lena Guisewite for her sewing skills, help with sampling, and all the fun times. Numerous student workers spent many hours helping with various aspects of these projects: Mindy Drewette, Natalia Czado, Colin Wong, and Chris Ackerman; thank you for contributing to my success. I thank the Department of Entomology and the Department of Biology for providing me the opportunity to teach, and the assistantships that came along with these opportunities. And thanks to my many friends, particularly the Friday Afternoon Seminar faithful, for all the good times we had together.

Finally, and most importantly, I would like to thank my parents, Mark and Janet Cammack, and grandparents, Joe and Helen Bennett, for all of their support during the past 10 years I have been a college student.

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER 1

CHAPTER 2

CHAPTER 5

LIST OF FIGURES

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

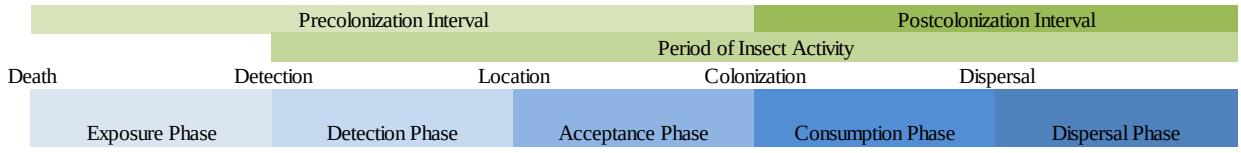
INTRODUCTION

In forensic cases involving entomological evidence, establishing the minimum postmortem interval (mPMI) is a critical component of the investigation that can help in case resolution (Catts and Goff, 1992). The ability to make this estimation operates under the premise that insects, particularly blow flies (Diptera: Calliphoridae), are the first insects attracted to decomposing remains and will oviposit on the remains shortly after death (Lord and Rodriguez, 1989; Catts, 1992). For lack of available human research subjects, much of the research in forensic entomology has been conducted using the pig model (Schoenly et al., 2006). Although not identical in every aspect, the pig model approximates a human quite well (Schoenly et al., 2007), and the variability in insect activity within and between pig-based studies is likely consistent with that in human decomposition. For example, oviposition on thawed pig carcasses has been observed within twenty minutes (Cammack, unpublished data), within an hour on freshly-killed pigs (Anderson and VanLaerhoven, 1996), and within six hours on frozen piglets (Payne, 1965). In addition, a number of extrinsic factors affect the rate at which these insects colonize remains (Campobasso et al., 2001); of particular interest are the availability of the remains and subsequent access to them.

Once a homicide or untimely death has occurred, perpetrators or other individuals may hide or conceal the remains in an attempt to delay or prevent their discovery. Although the concealment of remains is well-documented by case reports (e.g. Erzinçlioğlu, 1985, Benecke, 1998; Goff, 1992; Pujol-Luz et al., 2008), there has been little empirical research to quantify the effects of concealment on colonization rates. Wrapping remains in blankets delays colonization by 2.5 days (Goff, 1992), and concealing remains indoors delays

colonization between one day (Reibe and Madea, 2010) and five days (Anderson, 2011). The discovery of decomposing remains indoors is quite common (Goff 1991; Frost et al. 2011; Smeeton et al. 1984), and in such a situation, the remains might not be discovered until after the initial colonizing larvae have completed development and emerged as adults (Benecke et al., 2004 and Parker et al., 2010). A reliable method to age these adult flies is warranted, as current methods of estimating the mPMI are based on the duration of immature development and do not include the adults present at the scene. The ability to include this information in an entomological estimate of the mPMI would greatly enhance the application of forensic entomology to cases involving indoor remains (Erzinçlioğlu 1986; Catts and Goff, 1992).

The two afore-mentioned situations (how concealing remains affects colonization, and the discovery of adult flies at a scene) can be described in the context of the decomposition process as defined by Tomberlin et al. (2011) (Figure 1), and are represented



by the detection

Figure 1. Stages of the vertebrate decomposition process, with respect to insect activity. Modified from Tomberlin et al. (2011).

and acceptance phases and the very end of the dispersal phase. This framework was developed to provide distinct categories on which research could be based, and can be applied to decomposition research in any environment. Typically, forensic entomology

research focuses only on one of these phases, either the pre- or postcolonization interval, or a smaller fragment of one of these two phases.

For over 20 years, entomologists have recognized the need for research on how concealment of remains affects colonization (Erzinçlioğlu 1986; Catts, 1992; Amendt et al., 2004), and also how the presence of adult blow flies at an indoor scene can be utilized to the fullest extent (Erzinçlioğlu 1986; Catts and Goff, 1992). The following research is one of only a few studies to address decomposition in concealed environments, and is the first to present data that can be used to age adult blow flies collected at a scene. The goal of this study is to conduct the first of its kind to combine studies of the pre- and postcolonization intervals of remains located in indoor environments. Specifically, I will address how concealing remains affects the duration of the pre-CI across different seasons and concealment methods, and how a biochemical assay, the quantification of pteridines in the heads of blow flies, can turn an underutilized piece of evidence (adult blow flies collected at an indoor scene) into a tool that will aid in estimating the mPMI. An additional two chapters are included on methods for rearing blow flies in the laboratory and improving evidence analysis.

References

Amendt, J. R. Krettek, and R. Zehner. 2004. Forensic entomology. Naturwissenschaften 91: 51-65.

Anderson, G.S. 2011. Comparison of decomposition rates and faunal colonization of carrion in indoor and outdoor environments. Journal of Forensic Sciences 56: 136-142.

Anderson, G.S. and S.L. VanLaerhoven. 1996. Initial studies on insect succession on carrion in southwestern British Columbia. Journal of Forensic Sciences 41: 617-625.

Benecke M. 1998. Six forensic entomology cases: description and commentary. Journal of Forensic Sciences 43: 797-805.

Benecke M., E. Josepji, & R. Zweihoff. 2004. Neglect of the elderly: Forensic entomology cases and considerations. Forensic Science International 146S: S195-S199.

Campobasso, C.P. G. Di Vella, and F. Introna. 2001. Factors affecting decomposition and Diptera colonization. Forensic Science International 120: 18-27.

Catts, E.P. 1992. Problems in estimating the postmortem interval in death investigations. Journal of Agricultural Entomology 9: 245-255.

Catts, E.P. and M.L. Goff. 1992. Forensic entomology in criminal investigations. Annual Review of Entomology 37: 253-272.

Erzinçlioğlu, Y.Z. 1985. The entomological investigation of a concealed corpse. Medicine, Science, and the Law 25: 228-230.

Erzinçlioğlu, Y.Z. 1986. Areas of research in forensic entomology. Medicine, Science, and the Law 26: 273-278.

Frost, C.L. H.R. Braig, J. Amendt, and M.A. Perotti. 2011. Indoor arthropods of forensic importance: Insects associated with indoor decomposition and mites as indoor markers. In *Current Concepts in Forensic Entomology*, J. Amendt, M.L. Goff, C.P. Campobasso, and M. Grassberger, eds. Springer, Dordrecht, Netherlands. Pp 93-108.

Goff, M.L. 1991. Comparison of insect species associated with decomposing remains recovered inside dwellings and outdoors on the island of Oahu, Hawaii. Journal of Forensic Sciences 36: 748-753.

Goff, M.L. 1992. Problems in estimation of postmortem interval resulting from wrapping of the corpse: A case study from Hawaii. Journal of Agricultural Entomology 9: 237-243.

Lord, W.D. and W.C. Rodriguez, III. 1989. Forensic entomology: the use of insects in the investigation of homicide and untimely death. The Prosecutor 22: 41-48.

Parker, M.A. M. Benecke, J.H. Byrd, R. Hawkes, and R. Brown. 2010. Entomological alteration of bloodstain evidence. In *Forensic Entomology: The Utility of Arthropods in Legal Investigations*, J.H. Byrd and J.L. Castner, eds. CRC Press, Boca Raton, Florida, USA. Pp 539-380.

Payne, J.A. 1965. A summer carrion study of the baby pig *Sus scrofa* Linnaeus. Ecology 46: 592-602.

Pujol-Luz, J.R. P. Abdon do Costa Francez, A. Ururahy-Rodrigues, and R. Constantino. 2008. The black soldier fly, *Hermetia illucens* (Diptera, Stratiomyodae), used to estimate the postmortem interval in a case in Amapá State, Brazil. Journal of Forensic Sciences 53: 476-478.

Reibe, S. and B. Madea. 2010. How promptly do blowflies colonize fresh carcasses? A study comparing indoor with outdoor locations. Forensic Science International 195: 52-57.

Schoenly, K.G. N.H. Haskell, D.K. Mills, C. Bieme-Ndi, K. Larsen, andY. Lee. 2006. Recreating death's acre in the school yard: Using pig carcasses as model corpses to teach concepts of forensic entomology and ecological succession. The American Biology Teacher 68: 402-410.

Schoenly, K.G. N.H. Haskell, R.D. Hall, and J.R. Gbur. 2007. Comparative performance and complementarity of four sampling methods and arthropod preference tests from human and porcine remains at the forensic anthropology center in Knoxville, Tennessee. Journal of Medical Entomology 44: 881-894.

Smeeton, W.M.I. T.D. Koelmeyer, B.A. Holloway, and P. Singh. 1984. Insects associated with exposed human corpses in Auckland, New Zealand. Medicine, Science, and the Law 24: 167-174.

Tomberlin, J.K. R. Mohr, M.E. Benbow, A.M. Tarone, and S. VanLaerhoven. 2011. A roadmap for bridging basic and applied research in forensic entomology. Annual Review of Entomology 56: 401-421.

CHAPTER 1

Effects of concealment of small remains on colonization by insects

J.A. Cammack, A.C. Cohen, K.L. Kreitlow, R.M. Roe, and D.W. Watson

Key words: forensic entomology, decomposition, succession

Abstract

The decomposition of pig carrion (*Sus scrofa* (L.)) placed in concealed and open environments, and subsequent colonization by necrophagous insects, was studied during the spring, summer, and fall in Raleigh, North Carolina. Remains were concealed in three manners, ranging from minimal to well concealed, in simulated attics. Concealment had a significant effect on the insect community colonizing the remains. Although calliphorids and other fly species colonized remains located indoors, the beetles *Necrobia rufipes* and *Dermestes maculatus* were the only species indicative of remains located indoors. In contrast, numerous fly species, beetle species, and an ant species, were indicative of remains located outdoors. Season also significantly affected the insect species colonizing decomposing remains, particularly blow flies. *Lucilia illustris* was indicative of the spring, *Cochliomyia macellaria* and *Chrysomya rufifacies* were indicative of the summer, and *Calliphora vicina* and *Calliphora vomitoria* were indicative of the fall. Additionally, across all seasons, concealment of remains delayed colonization by 35-768 hours, depending on the degree of concealment. These differences between the insect communities across seasons and concealment treatments, and the effects of concealment on colonization indicate that such information is relevant and should be considered when analyzing evidence for criminal investigations.

Introduction

The body of literature on decomposition ecology is vast and of great use in homicide investigations (e.g. Anderson & VanLaerhoven, 1996; Benbow et al., 2013; Hobischak et al., 2006; Payne, 1965; Reed, 1958; Tabor et al. 2004, 2005; Tomberlin and Adler, 1998; Watson and Carlton, 2003). Many of these studies have characterized duration of the pre-colonization interval, the rate of decay, and the insect species (baseline fauna) and succession patterns associated with carrion in a particular environment, location, or season, which can be useful for estimating the minimum postmortem interval (mPMI) (Schoenly et al., 1996). Because of the difficulties presented in using human remains, most studies utilize swine (*Sus scrofa* L.) weighing > 23 kg to model the adult human corpse (Schoenly et al. 2006). However, few studies have been conducted using smaller pig carcasses (Payne, 1965; Benbow et al., 2013), and none have done so to model juvenile remains.

The homicide rate of juveniles is substantially higher in the United States than any other developed country, with approximately 1,800 juvenile homicide victims in 1999, and is the only cause of childhood death that has increased in incidence over the past 30 years (Finkelhor and Ormrod, 2001). Homicide is ranked in the top four causes of juvenile mortality (ranked second, third, or fourth depending on age group), and significantly more children under the age of four die of homicide than of infectious disease or cancer (Heron et al., 2009). In a survey of 162 child fatality cases from North Carolina, the peak age categories of death from maltreatment were 0-3 months (25%) and 2-6 years (19%) (Ross et al. 2009). The small size of child remains makes them easily concealed to prevent discovery. Juvenile remains have been found hidden indoors in containers, wrapped in blankets, or

outside in garbage bags (Pujol-Luz et al., 2008). This concealment not only delays discovery by investigators, but also by insects, thus increasing the duration of the precolonization interval. Insect colonization of remains concealed indoors is delayed in comparison to those located outdoors, and indoor remains are colonized by different insect species (Anderson, 2011; Goff, 1991; Reibe & Madea 2010), and access of the remains to insects is the second most important variable affecting the rate of decomposition (Mann et al., 1990).

Decomposition studies using the pig model have been conducted various eastern states, in South Carolina (Payne 1965, 1972), Tennessee (Shahid et al., 2003) and Virginia (Tabor et al., 2004, 2005), but this is the first to do so in North Carolina. Therefore, the goals of this study are 1) to identify the local insect species of forensic importance that colonize child-sized remains during the spring, summer, and fall; and 2) determine how concealment of child-sized remains affects colonization patterns of these insects and the subsequent decomposition.

Materials and Methods

Study Design

Experiments were conducted at the North Carolina State University Lake Wheeler Road Field Laboratory, in Raleigh, North Carolina. Two experiments were conducted during the spring (18 April – 11 May 2011, 23 May – 19 June 2012), summer (28 August – 8 September 2010, 12 – 25 July 2011), and fall (15 October – 24 December 2010, 20 October – 21 December 2011). Five pig carcasses ranging from 5.44-14.5 kg (mean ± SE: 10.2 ± 0.51 kg) were used in each seasonal study, for a total of 30 carcasses. Carcasses were obtained frozen from the NCSU Swine Education Unit and were either natural deaths or

euthanized by captive bolt pistol by farm staff, for reasons unknown. Before being placed in the field, the carcasses were thawed at approximately 22.5°C for approximately 60 hours in a dark, sealed room with no insect access. For each seasonal replicate the thawed carcasses were placed *in situ* approximately 1.5 hr after morning civil twilight, and the first observation was taken 2 hr after the morning civil twilight.

To study the effect of concealment on decomposition, three simulated attics were constructed in an abandoned, gable-roof poultry house with open sides. The attics were located approximately 4.9 m apart within the structure. Each attic was 4.86 m^2 and had 2 doors; which, when opened, provided a 0.88 m^2 opening through which the carcasses could be monitored and sampled. To provide air exchange within the attics and potential entry points for insects, circular holes 9 cm in diameter were cut into each sides of the attics (soffit vents, 3 holes per side) and at the peak of the attic above the door (gable vents, 2 holes per attic). Commercially-available aluminum soffit and gable vents were placed over the holes on each side of the attic, and above the upper door to resemble the ventilation that would be found in a home. The floor of each attic had a removable wooden panel approximately 1.22 x 1.22 m. Floor panels were replaced at the beginning of each season because fluids associated with decomposition of the remains soaked the wood (Figures 1 and 2). Before each seasonal replicate began, the attics were thoroughly cleaned; the pig bones and other dried tissue and insect remnants (empty puparia) were removed, the attics vacuumed, any attic structures that absorbed decomposition fluids aside from the removable floor were sanded and sealed with white, latex paint. Within each season (spring (SP), summer (SU), and fall FA)), carcasses were concealed within the attics in one of three manners, representing minimal, moderate and

well concealed. The BOX treatment was considered well concealed. For this treatment, the carcass was placed in a 113.5 L trash bag (Hefty® CinchSak®) which was then placed inside a 113.5 L (77.5 x 46.4 x 46.4 cm) Rubbermaid® storage bin with a lid and placed in the attic. The drawstrings on the trash bag were pulled tight, and tied in an overhand knot. To allow access for sampling, an oval-shaped door, ~51 x 17.5 cm, was installed in the side of the storage bin. The bottom of the plastic bag was cut and sealed to the side of the container with latex caulking and a wooden bezel on which the door articulated. The door was sealed with a rubber gasket and a latch to prevent insect access through the door. For the moderately-concealed treatment (BLANKET), the carcass was wrapped "burrito-style" in a 127 x 152 cm polyester, fleece throw blanket and placed on the attic floor. A 72 cm long zipper was sewn longitudinally into the middle of the blanket to allow access to the remains without compromising the wrapping of the remains. The third attic treatment (EXPOSED) had no additional concealment and served as a minimal positive control for the other attic treatments, and the carcass was placed on the attic floor. Treatments were randomly assigned to the attic with each replicate.

Two additional carcasses were placed outside in natural conditions, approximately 112 m south of the attics, and served as controls. One carcass was placed in an open field (SUN treatment) which had daily direct exposure for the duration of each study period. Plant species in the field include: Bermuda grass (*Cynodon dactylon* (L.) Pers.), wild garlic (*Allium vineale* L.), Carolina geranium (*Geranium carolinianum* L.), buckhorn plantain (*Plantago lanceolata* L.), clover (*Trifolium* sp.), *Galium aparine* L. (Rubiaceae), and Fabaceae sp. The other carcass was placed in a shaded environment (SHADE), which was shaded by sweetgum

(*Liquidambar styraciflua* L.) oak (*Quercus* sp.), and loblolly pine (*Pinus tadea* L.) trees, and a spruce wood lattice "roof", located approximately 2.4 m above the ground. Carcasses were protected from scavenging by vertebrates with cages constructed of galvanized wire mesh and PVC pipe. The SUN and SHADE sites were approximately 50 m apart.

Figure 1. External view of one of the simulated attics, with both doors closed.

Figure 2. External view of one of the attics with both doors open. The removable floor panel is partially removed and enclosed within the circle.

Sampling Protocol

During each season, all five carcasses were monitored twice daily (approximately two hours after morning civil twilight and two hours before evening civil twilight, respectively) for insect activity. Carcasses were sampled at this interval until all blow fly larvae had dispersed from the remains, or the next calendar season had begun. During inclement weather (e.g. rain), only the attic carcasses were sampled to prevent damage to sampling equipment. At each sampling time, the temperatures of the remains and surrounding environment were recorded for each carcass, but varied by treatment (Table 1). A Thermolyne® digital pyrometer with at type K thermocouple and a Cen-Tech™ infrared thermometer were used to measure temperatures.

Table 1. Locations of the temperatures recorded at each of five carcasses.

	Temperature Reading				
Carcass	Ambient	Body Surface	Beneath Remains[1]	Soil[2]	Maggot Mass[3]
BOX	X	X	-	-	X
BLANKET	X	X	X	-	X
EXPOSED	X	X	X	-	X
SUN	X	X	X	X	X
SHADE	X	X	X	X	X

[1] Temperatures were not recorded beneath the pig in the BOX treatment to prevent puncturing the plastic bag with the temperature probe.
[2] Taken at a depth of ~10 cm, ~ 0.5 m away from the remains.
[3] Temperatures of each distinct maggot mass were recorded.

The presence of insects was noted at each sampling interval, and species were visually identified to the lowest taxonomic level possible. Adult insects were collected from all carcasses by sweep net or modified Dust Buster® hand-held vacuum (for flies) or by hand

(beetles) during all sampling times, except for the first time when adult flies were noticed on the remains. Insects were only visually identified but not collected at this time so that the natural colonization of the remains was not compromised. During the morning sampling period, fly larvae (~50-100 individuals) were collected by hand with soft forceps or a plastic spoon and transported to the laboratory where they were fixed in sub-boiling (≤100°C) water and then preserved in 80% EtOH in 4 dram vials. During the evening sampling period, fly larvae (~50-100 individuals) were collected by hand with forceps or a plastic spoon and placed in an aluminum foil pouch containing beef heart, kidney, or liver (Byrd et a., 2010). The foil pouch was then placed in a 708 ml GladWare® Soup & Salad container which had 1-2 cm of vermiculite or soil in the bottom for a pupation substrate. The lid of each container was perforated with 10 holes approximately 1 mm in diameter to allow for air flow but prevented the escape of the larvae. The containers of larvae were held at room temperature (~22.5°C) until adult emergence. The containers were then stored frozen (-20°C) until the insects were removed for identification.

Hobo® H8 relative humidity and temperature data loggers (Onset Computer Corporation, Bourne, MA, USA) recorded hourly temperature and humidity inside each attic and at the SHADE location. At the SUN location, two temperatures were recorded hourly with a Hobo® H8 temperature/external channel data logger (Onset Computer Corporation, Bourne, MA, USA). The data logger was placed in an enclosure with an open bottom to prevent sun exposure from elevating the recorded ambient temperature, and an external probe was placed beside the pig carcass to record temperatures in direct sunlight.

Data were analyzed with non-metric multidimensional scaling (NMDS), multiple response permutation procedure (MRPP), and indicator species analysis (ISA) in the statistical package R version 3.0.0. Community dissimilarity was evaluated using the Bray-Curtis dissimilarity index.

Results

Nonmetric multidimensional scaling (NMDS) analyses indicated that two ordinations were sufficient for describing insect communities across all seasons and treatments. Multiple response permutation procedures (MRPP) indicated that the community composition of insect species visiting decomposing pigs was significantly different between seasons ($p = 0.001$, Δ: Spring= 0.7831 , Summer=0.7894, Fall= 0.8072, A= 0.03). Subsequent indicator species analysis (ISA) indicated that 16 of the 31 most common taxa were significant indicators of a given season (Table 2). The black blow fly, *Phormia regina* Meigen, was the most frequently encountered species (present at 40% of all sampling events), and occurred at all carcasses during all three seasons of both years. Although an indicator species for spring, the blow fly *Lucilia illustris* Meigen was present during both the spring and fall, and was captured at carcasses from mid October to late May. The warm-weather species *Cochliomyia macellaria* and *Chrysomya megacephala* were indicative of the summer but were collected at carcasses from late April to late October and mid July to mid November, respectively. *Calliphora vicina* and *C. vomitoria* were collected from mid October to mid April (during the cooler portions of the year), but were indicative of the fall.

Table 2. Indicator insect taxa for each of three seasons, α=0.05.

Season	Insect Taxon	Indicator Value	P value
Spring	*Lucilia illustris*	0.0577	0.001
	Hydrotaea aenescens	0.2	0.001
	Fannia sp.	0.191	0.001
	Phoridae sp.	0.139	0.001
	Oiceoptoma inequale	0.117	0.001
	Oiceoptoma novaboracense	0.117	0.001
	Dermestes maculatus	0.156	0.001
Summer	*Cochliomyia macellaria*	0.246	0.001
	Chrysomya megacephala	0.044	0.009
	Musca domestica	0.154	0.001
	Prochyliza xanthostoma	0.0811	0.019
	Sepsidae sp.	0.0904	0.001
	Necrophila americana	0.127	0.003
	Necrobia rufipes	0.0812	0.009
	Histeridae sp.	0.0453	0.023
Fall	*Calliphora* spp.	0.039	0.023
	Calliphora vicina		
	Calliphora vomitoria		

For all three seasons, MRPP analyses indicated that the community composition of insect species visiting the remains differed significantly based on treatment ($p = 0.001$ for all three seasons) (Table 3). Although a number of species were common to each carcass treatment within a given season (Figures 3-5), ISA revealed that 18, 11, and 11 species were significant indicators of a given carcass treatment, within spring, summer, and fall, respectively (Tables 4-6).

Table 3. Bray-Curtis dissimilarity values (Δ) for each carcass treatment within each season, and MRPP statistics for each season.

	Carcass Treatment					MRPP Statistics			
Season	BOX	BLANKET	EXPOSED	SUN	SHADE	ΔObserved	ΔExpected	A	P value
Spring	0.787	0.608	0.741	0.71	0.628	0.688	0.783	0.122	0.001
Summer	0.802	0.779	0.691	0.663	0.601	0.71	0.789	0.1	0.001
Fall	0.707	0.764	0.753	0.792	0.775	0.759	0.807	0.06	0.001

Multiple blow fly species visited and colonized all three indoor treatments (Table 7). However, MRPP and ISA indicated that the insect community associated with indoor remains was significantly different from that associated with outdoor remains, during all three seasons (Figures 6-8 and Tables 8-10). The beetles *Necrobia rufipes* (DeGeer) (Cleridae) and *Dermestes maculatus* (DeGeer) (Dermestidae) were indicative of remains located indoors, while most species indicative of outdoor remains were flies. False honey ants (*Prenolepis* sp.) were indicative of outdoor remains and were present and consumed fly eggs (Figure 9) and larvae throughout much of the decomposition process of carcasses placed at the shade location. The species was seen at carcasses placed in the shade for all replicates except for the first summer. Although carrion beetle adults were noted visiting carcasses placed in all locations, they were indicator species of outdoor carcasses. The most abundant species, *Necrophila americana* was an indicator species of carcasses placed in the shade during the spring and summer. Adults of the species were frequently encountered at the shade carcass where they congregated in large numbers to mate and feed on the fly larvae consuming the remains (Figure 10). Although the species was not seen during the fall of year 2, it was likely present, just not encountered, as adults were present at the carcass placed in the sun, which was located approximately 50 m west of the shade carcass.

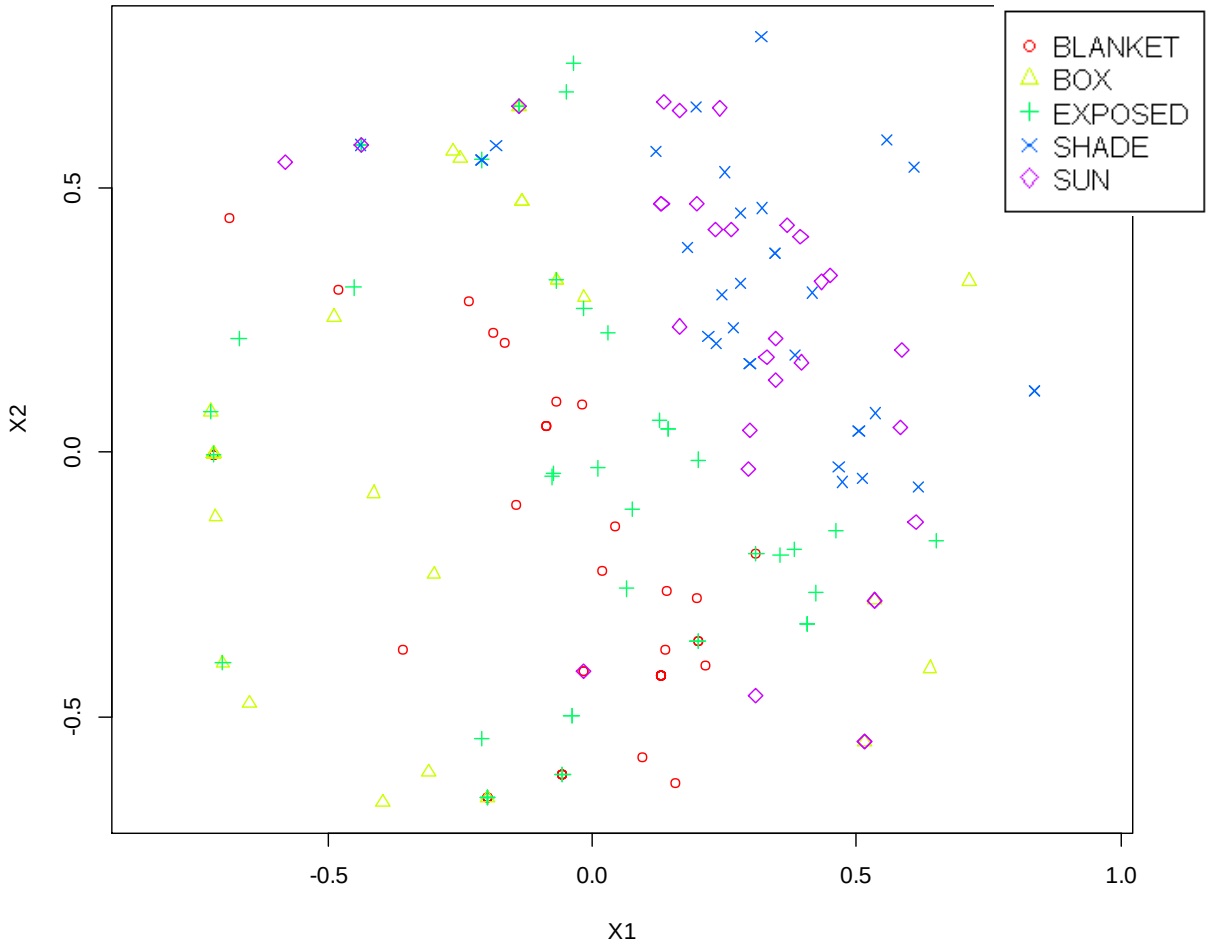


Figure 3. NMDS ordination of the insect community structure across carcass treatments, for the spring.

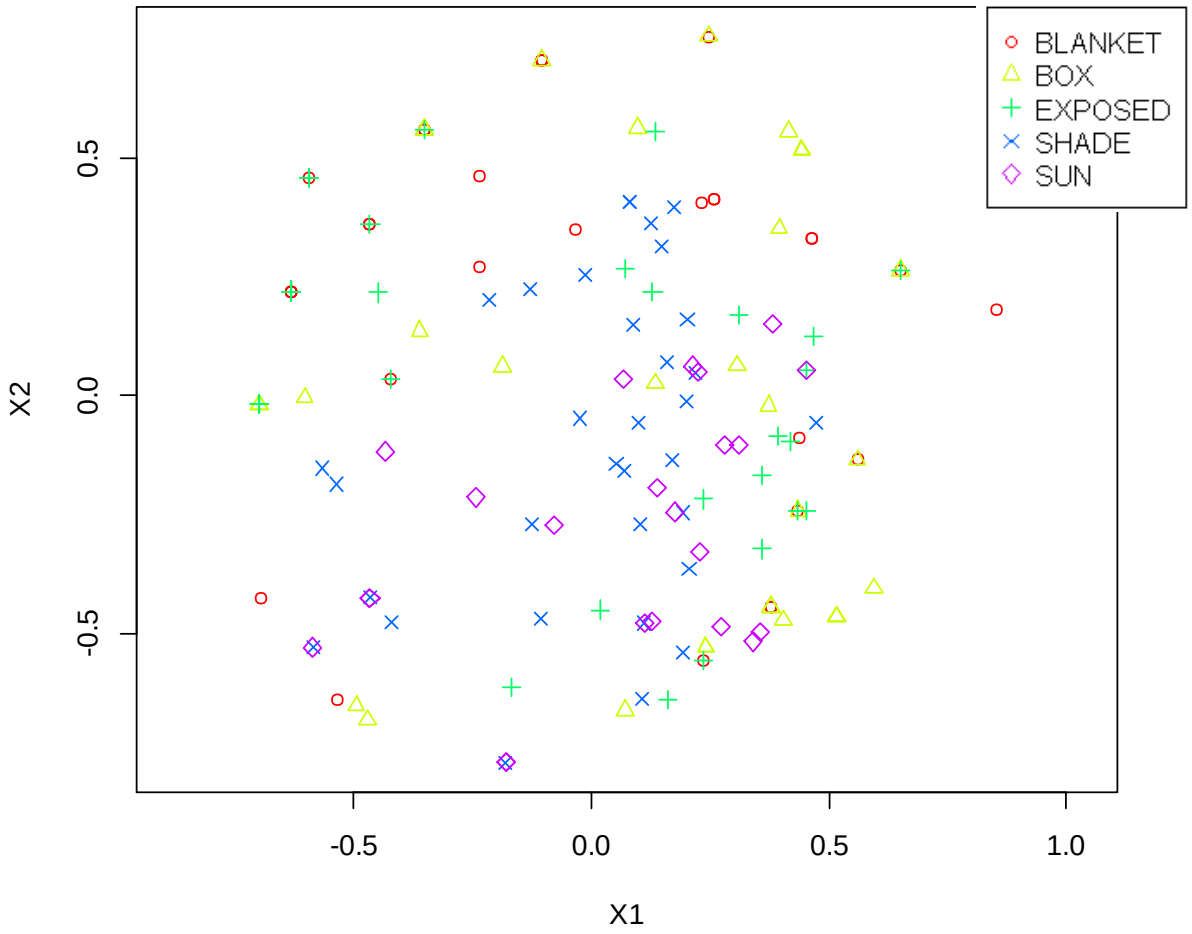


Figure 4. NMDS ordination of the insect community structure across carcass treatments, for the summer.

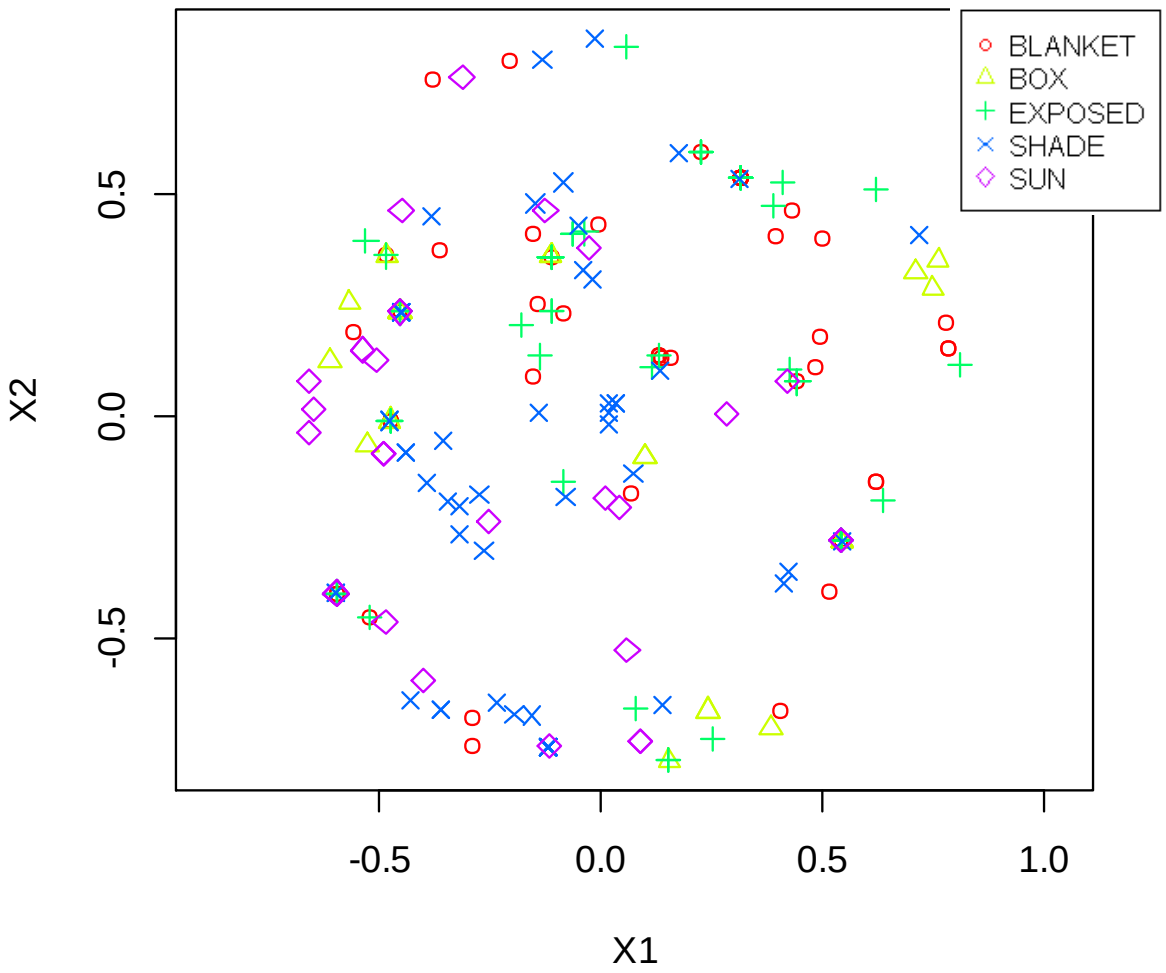


Figure 5. NMDS ordination of the insect community structure across carcass treatments, for the fall.

Table 4. Indicator insect tax for each of five carcass treatments during the spring, α=0.05.

Carcass Treatment	Insect Taxon	Indicator Value	P value
BOX	Calliphoridae sp.	0.078	0.031
	Phoridae sp.	0.206	0.031
BLANKET	*Lucilia sericata*	0.11	0.007
	Musca domestica	0.164	0.006
	Necrobia rufipes	0.247	0.001
	Dermestes maculatus	0.364	0.001
EXPOSED	*Lucilia sericata*	0.11	0.007
	Musca domestica	0.164	0.006
	Necrobia rufipes	0.247	0.001
	Dermestes maculatus	0.364	0.001
SUN	*Lucilia coeruleiviridis*	0.189	0.027
	Lucilia illustris	0.214	0.003
	Calliphora spp.	0.0724	0.036
	Calliphora vicina		
	Calliphora vomitoria		
	Sarcophagidae sp.	0.0668	0.035
	Sepsidae sp.	0.0812	0.008
	Oiceoptoma inequale	0.178	0.001
	Histeridae sp.	0.21	0.001
SHADE	*Phormia regina*	0.183	0.021
	Lucilia coeruleiviridis	0.103	0.027
	Lucilia illustris	0.0466	0.003
	Calliphora spp.	0.004	0.036
	Calliphora vicina		
	Calliphora vomitoria		
	Prochyliza xanthostoma	0.255	0.001
	Necrophila americana	0.411	0.001
	Oiceoptoma inequale	0.176	0.001
	Oiceoptoma novaboracense	0.207	0.001
	Histeridae sp.	0.011	0.001
	Formicidae (*Prenolepis* sp.)	0.265	0.001

Table 5. Indicator insect tax for each of five carcass treatments during the summer, α=0.05.

Carcass Treatment	Insect Taxon	Indicator Value	P value
BOX	N/A	N/A	N/A
BLANKET	N/A	N/A	N/A
EXPOSED	Phoridae sp.	0.0657	0.073
	Necrobia rufipes	0.224	0.002
	Dermestes maculatus	0.151	0.019
SUN	*Phormia regina*	0.189	0.027
	Cochliomyia macellaria	0.214	0.003
SHADE	*Lucilia coeruleiviridis*	0.172	0.003
	Hydrotaea aenescens	0.197	0.007
	Prochyliza xanthostoma	0.227	0.001
	Sepsidae sp.	0.273	0.001
	Necrophila americana	0.431	0.001
	Histeridae sp.	0.152	0.004
	Formicidae (*Prenolepis* sp.)	0.424	0.001

Table 6. Indicator insect tax for each of five carcass treatments during the fall, α=0.05.

Carcass Treatment	Insect Taxon	Indicator Value	P value
BOX	*Phormia regina*	0.125	0.43
	Necrobia rufipes	0.124	0.001
	Dermestes maculatus	0.055	0.038
BLANKET	*Necrobia rufipes*	0.124	0.001
	Dermestes maculatus	0.055	0.038
EXPOSED	*Hydrotaea aenescens*	0.17	0.006
	Necrobia rufipes	0.124	0.001
	Dermestes maculatus	0.055	0.038
SUN	*Lucilia coeruleiviridis*	0.177	0.001
	Lucilia illustris	0.0696	0.018
	Hydrotaea aenescens	0.17	0.006
	Necrophila americana	0.248	0.001
SHADE	*Cochliomyia macellaria*	0.118	0.002
	Calliphora spp.	0.139	0.001
	Calliphora vicina		
	Calliphora vomitoria		
	Histeridae sp.	0.0841	0.006
	Formicidae (*Prenolepis* sp.)	0.306	0.001

Table 7. Blow fly species reared from decomposing porcine remains from all five treatments and three seasons.

		Blow Fly Species											
	Carcass Treatment	*Phormia regina*	*Cochliomyia macellaria*	*Chrysomya megacephala*	Chrysomyinae spp.	*Lucilia coeruleiviridis*	*Lucilia sericata*	*Lucilia cuprina*	*Lucilia illustris*	*Lucilia* spp.	*Calliphora vicina*	*Calliphora vomitoria*	*Calliphora* spp.
Spring	BOX	X	X		X		X	X		X			
	BLANKET	X	X				X			X			X
	EXPOSED	X	X	X	X		X			X			X
	SUN	X	X				X			X			X
	SHADE	X	X		X					X			X
Summer	BOX	X	X	X	X		X			X			
	BLANKET	X	X	X	X		X			X			
	EXPOSED	X	X		X		X			X			
	SUN	X	X		X					X			
	SHADE	X	X		X								
Fall	BOX	X	X	X	X		X			X	X		X
	BLANKET	X		X	X		X			X			
	EXPOSED	X	X		X		X	X		X	X		X
	SUN	X	X		X	X			X	X	X		X
	SHADE	X	X		X		X					X	X

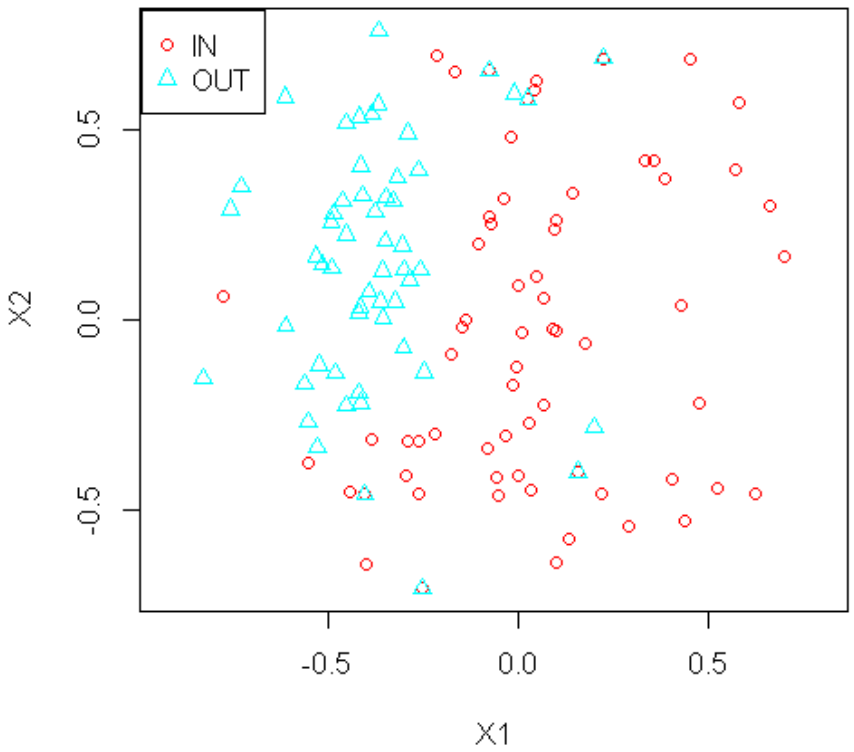


Figure 6. NMDS ordination of the insect community structure associated with decomposing remains located in indoor and outdoor environments during the spring.

Table 8. Indicator insect taxa for carcasses located indoors or outdoors during the spring, α=0.05.

Carcass Location	Insect Taxon	Indicator Value	P value
Indoors	*Necrobia rufipes*	0.252	0.001
	Dermestes maculatus	0.359	0.001
	Phoridae sp.	0.282	0.001
Outdoors	*Phormia regina*	0.425	0.001
	Lucilia coeruleiviridis	0.232	0.001
	Lucilia illustris	0.158	0.001
	Prochyliza xanthostoma	0.385	0.001
	Creophilus maxillosus	0.266	0.015
	Necrophila americana	0.662	0.001
	Oiceoptoma inequale	0.354	0.001
	Oiceoptoma novaboracense	0.354	0.001
	Histeridae sp.	0.154	0.001
	Formicidae (*Prenolepis* sp.)	0.139	0.001

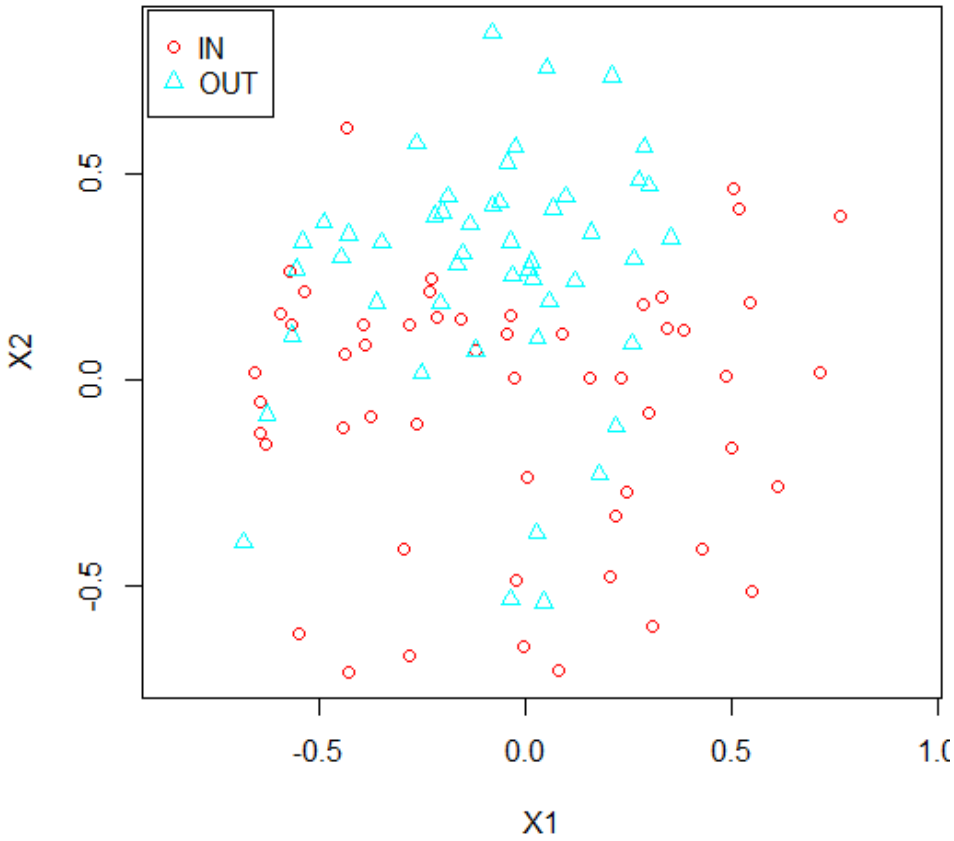


Figure 7. NMDS ordination of the insect community structure associated with decomposing remains located in indoor and outdoor environments during the summer.

Table 9. Indicator insect taxa for carcasses located indoors or outdoors during the summer, α=0.05.

Carcass Location	Insect Taxon	Indicator Value	P value
Indoors	*Necrobia rufipes*	0.248	0.001
Outdoors	*Cochliomyia macellaria*	0.3	0.012
	Chrysomya megacephala	0.161	0.002
	Lucilia coeruleiviridis	0.205	0.001
	Prochyliza xanthostoma	0.293	0.001
	Sepsidae sp.	0.304	0.001
	Necrophila americana	0.732	0.001
	Histeridae sp.	0.25	0.001
	Formicidae (*Prenolepis* sp.)	0.25	0.001

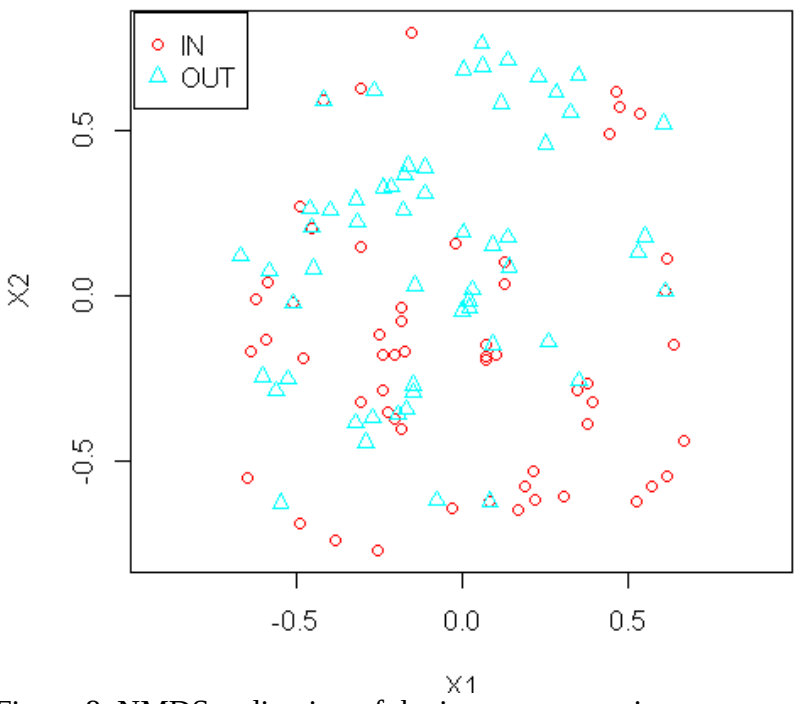


Figure 8. NMDS ordination of the insect community structure associated with decomposing remains located in indoor and outdoor environments during the fall.

Table 10. Indicator insect taxa for carcasses located indoors or outdoors during the fall, α=0.05.

Carcass Location	Insect Taxon	Indicator Value	P value
Indoors	Calliphoridae sp.	0.052	0.03
	Hydrotaea aenescens	0.238	0.01
	Creophilus maxillosus	0.203	0.048
	Dermestes maculatus	0.052	0.024
Outdoors	*Phormia regina*	0.3	0.021
	Cochliomyia macellaria	0.183	0.001
	Chrysomya megacephala	0.108	0.002
	Lucilia coeruleiviridis	0.177	0.001
	Lucilia illustris	0.0652	0.009
	Lucilia sp.	0.156	0.009
	Calliphora sp.	0.183	0.001
	Prochyliza xanthostoma	0.0891	0.045
	Necrophila americana	0.151	0.001
	Histeridae sp.	0.081	0.003
	Formicidae (*Prenolepis* sp.)	0.226	0.001

Figure 9. False honey ants (*Prenolepis* sp.) (circled) consuming blow fly eggs (*Lucilia* sp. and *Phormia regina*) in the mouth of a decomposing pig during spring of year one.

Figure 10. American carrion beetles, *Necrophila americana* (L.) consuming fly larvae at a decomposing pig during spring of year one.

A positive relationship between the degree of concealment and the amount of time between first blow fly contact and subsequent colonization was noted during each season, except during spring 1 (Table 11, Figure 11). During SP1, flies made contact with the BOX pig approximately 24 hours before the BLANKET pig. Eggs or larvae were first collected from the BOX pig approximately 9 hours before the BLANKET pig. Except for F2, adult blow flies first visited the remains placed in the sun and shade within 10 hours or placement. In three of the replicates (SP2, SU1, and SU2), flies visited the remains less than one hour after placement in the field. In SP2, blow flies landed on the remains within 30 seconds of placement (S.S. Denning, personal communication). During SU1 and SU2, blow flies landed on the remains within 1 hour of placement and 20 minutes of placement, respectively, and eggs and larvae were collected approximately 11 hours later. During all seasons, *Lucilia* sp. (mainly *L. coeruleiviridis*) were the first to visit and colonize the outdoor remains; and for all seasons but spring and FA2, *P. regina* was also noted colonizing the remains at the same time.

Table 11. Amount of time (hours) between carcass placement and first recorded contact by adults and subsequent colonization (presence of eggs or larvae) for both study years.

		Year 1		Year 2	
	Carcass Treatment	Adults Present	Immatures Present	Adults Present	Immatures Present
Spring	BOX	153	168	107	120
	BLANKET	177	177	83	83
	EXPOSED	72	82	35	35
	SUN	10	10	0	24
	SHADE	10	10	0	24
Summer	BOX	72	72	48	72
	BLANKET	58	58	24	48
	EXPOSED	34	48	11	24
	SUN	10	10	0.3	11
	SHADE	10	10	0.3	0.3
Fall	BOX	153	153	751	768
	BLANKET	104	104	96	120
	EXPOSED	56	104	79	96
	SUN	7	8	31	55
	SHADE	8	8	31	31

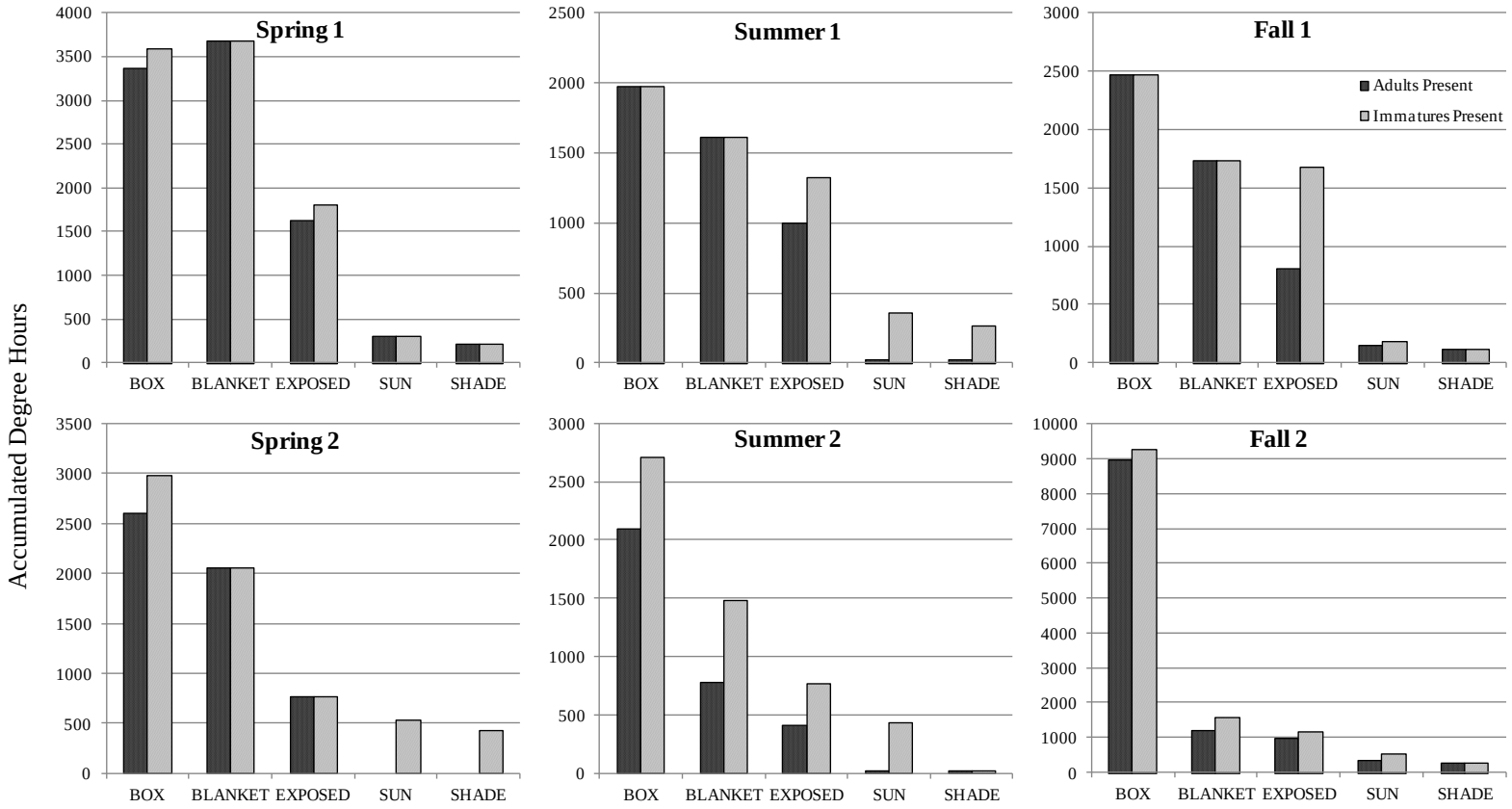


Figure 11. Accumulated degree hours between carcass placement and first contact by adult blow flies, and carcass placement and when eggs or larvae were first observed.

Discussion

The objectives of this study were to document the insect species attracted to small remains, and to quantitatively determine if season or concealment influenced the community structure of the insects visiting and colonizing decomposing remains. The season during which remains decomposed greatly influenced the insect species, particularly the blow flies that visited and colonized the remains. Results from our study corroborate the findings of other seasonal decomposition studies conducted in South Carolina (Tomberlin and Adler, 1998), Tennessee (Reed, 1958), and in Virginia (Tabor et al., 2004, 2005). Previously-published information indicates that *Lucilia illustris* is a species most active during the warm summer months (Byrd and Castner, 2009), but was an indicator species of spring in our study. Our findings also indicate that this species prefers carrion in bright or sunlit environments, supporting Byrd and Castner (2010). The calliphorid species indicative of summer (*C. macellaria* and *Ch. megacephala*) were expected to occur during this season, but the occurrence of *Ch. megacephala* into mid November was not expected. *Cochliomyia macellaria* has been collected in South Carolina from mid July to early September (Tomberlin and Adler, 1998) and as late as mid November (Cammack and Nelder, 2010); in Tennessee from mid May to mid November (Reed, 1958), and in from July to August in Virginia (Tabor et al., 2005). Much less is known about the seasonal activity of the exotic *Ch. megacephala*, but perhaps like its invasive congener *Ch. rufifacies*, the species might be acclimatizing to cooler temperatures as it spreads throughout North America (Cammack and Nelder, 2010). The cool-weather activity of *Calliphora vicina* and *Calliphora vomitoria* was expected and supports the findings of Tomberlin and Adler (1998), Reed (1958), and

Tabor et al. (2004, 2005). The year-round activity of *P. regina* and *Lucilia coeruleiviridis* and status as the first colonizers of decomposing remains from spring to fall indicates that future research should focus on these two species, particularly *L. coeruleiviridis*, a species for which no development data are available.

Carcass treatment also influenced the insect community associated with the remains. During the spring, *Lucilia sericata* was an indicator species of remains wrapped in a blanket or exposed in an attic. Although the species readily colonized remains indoors, it was not an indicator of remains located indoors. Colonizing remains indoors contradicts the findings of Reibe and Madea (2010), where *L. sericata* only entered a structure and oviposited in one of nine study replicates, but supports the findings of Anderson (2011). The species indicative of indoor remains across seasons (*Necrobia rufipes* and *Dermestes maculatus*) likely were able to occur with such a high frequency because of the large amount of resource available to them in the attics. Outdoors, the remains were colonized by such large numbers of calliphorids that all or nearly all soft tissue was consumed; whereas indoors, a lower number of larvae colonized the remains and the BLANKET and EXPOSED treatments mummified, becoming attractive for the two beetle species. The piophilid *Prochyliza xanthostoma* (Walker) was an indicator of outdoor remains during all seasons, and has been found on carrion in Tennessee year round (Reed, 1958) and during the spring and summer in Virginia (Tabor et al., 2004). The forensic importance of this species is likely underestimated, thus more studies on this species are warranted, as it is quite common at remains but no development data exist.

The positive relationship between degree of concealment and time to colonization was expected. However, the extremely long delay of colonization in the fall 2 study is interesting. Although the mean temperatures of the attics only differed by approximately 2°C between the study years, mean outdoor ambient temperatures differed by almost 6°C, suggesting that the volatiles that attract blow flies were likely negatively impacted by the cooler outdoor temperatures. In our indoor treatments, colonization occurred much sooner in comparison with Anderson (2011), but this is likely because our carcasses were enclosed in a smaller structure (simulated attic vs. a house), and were closer to the point of ventilation/ insect access. In contrast, pigs located in front of an open window were colonized 24 hours after placement in 7 of 9 experiments (Reibe and Madea, 2010); this only occurred during SU2 of our study. Containment of decomposition odors within the attics delayed visitation, but once odors began to escape, the attracted flies readily entered the attics. Once inside the attic, flies quickly colonized the EXPOSED remains, and overcame the BLANKET barrier by ovispositing outside the blanket or navigating through the blanket folds to reach the carcass. Similarly, flies entered the BOX through the holes at the handles (approximately 0.6 cm in diameter). Once inside the box, the flies navigated through the dark to find the top opening of the trash (where the bag was loosely tied), and gained access to the remains, as indicated by the presence of adults on the remains when making observations. Across all seasons, the difference between when the EXPOSED and BLANKET remains were colonized ranged from 0.5-3 days. Flies took 2.5 days to gain access to porcine remains wrapped in two layers of blankets in Hawaii (Goff, 1992). These results show that concealing remains in attics does delay colonization, which must be taken into consideration if remains

are found in such locations.

This study represents the first of its kind to characterize the insect fauna associated with decomposing remains in North Carolina, and is the first to use small pigs as a model for juvenile remains. We were able to use statistical analyses to find significant differences between the insect communities across season and concealment treatments. To our knowledge, this study is the first to use NDMS, MRPP, and ISA statistical analyses to evaluate the differences between the insect communities colonizing carrion located indoors vs. outdoors, and is the second to use these analyses for describing seasonal differences of the insect communities colonizing remains (Benbow et al., 2013). The differences we found indicate that such information needs to be considered when analyzing evidence for investigations. Additionally, we determined that *Phormia regina* is the most abundant and frequently-encountered blow fly species from March to November, and therefore likely the most important blow fly species for forensic investigations in North Carolina. Additionally, the common occurrence of *Lucilia coeruleiviridis* and *Prochyliza xanthostoma* suggests that further work on these species is warranted to recognize their full potential in forensic investigations.

Acknowledgement

We thank Steve Denning for design and construction of the attics, assistance with nearly every aspect of the study implementation, and assistance with data and sample collection; Lena Guisewite for assistance with data and sample collection, Dr. Jen Pechal for assistance with statistical analyses, Natalia Czado, Mindy Drewette, and Chris Ackerman for processing of larval specimens, and Colin Wong for help with construction of the attics.

References

Anderson, G.S. 2011. Comparison of decomposition rates and faunal colonization of carrion in indoor and outdoor environments. Journal of Forensic Sciences 56: 136-142.

Anderson, G.S. and S.L. VanLaerhoven. 1996. Initial studies on insect succession on carrion in southwestern British Columbia. Journal of Forensic Sciences 41: 617-625.

Benbow, M.E. A.J. Lewis, J.K. Tomberlin, and J.L. Pechal. 2013. Seasonal necrophagous insect community assembly during vertebrate carrion decomposition. Journal of Medical Entomology 50: 440-450.

Byrd, J.H. and J.L. Castner. 2010. Insects of Forensic Importance. In *Forensic Entomology: The Utility of Arthropods in Legal Investigations*, 2nd Edition. J.H. Byrd and J.L. Castner, eds. CRC Press, Boca Raton, Florida. 681 pp.

Byrd, J.H. W.D. Lord, J.R. Wallace, and J.K. Tomberlin. 2010. Collection of entomological evidence during legal investigations. In *Forensic Entomology: The Utility of Arthropods in Legal Investigations*, 2nd Edition. J.H. Byrd and J.L. Castner, eds. CRC Press, Boca Raton, Florida. 681 pp.

Cammack, J.A. and M.P. Nelder. 2010. Cool-weather activity of the forensically important hairy maggot blow fly Chrysomya rufifacies (Macquart) (Diptera: Calliphoridae) on carrion in Upstate South Carolina, United States. Forensic Science International 195: 139-142.

Finkelhor, D. and R.K. Ormrod. 2001. Offenders incarcerated for crimes against juveniles. Juvenile Justice Bulletin *NCJ191028. 12 pp.*

Goff, M.L. 1991. Comparison of insect species associated with decomposing remains recovered inside dwellings and outdoors on the island of Oahu, Hawaii. Journal of Forensic Sciences 36: 748-753.

Goff, M.L. 1992. Problems in estimation of postmortem interval resulting from wrapping of the corpse: A case study from Hawaii. Journal of Agricultural Entomology 9: 237-243.

Heron, M. D.L. Hoyert, S.L. Murphy, J. Xu, K.D. Kochanek, and B. Tejada-Vera. 2009. Deaths: Final Data for 2006. U.S. National Center for Health Statistics, National Vital Statistics Report 57 (14).

Hobischak, N.R. S.L. VanLaerhoven, and G.S. Anderson. 2006. Successional patterns of diversity in the insect fauna on carrion in sun and shade in the Boreal Forest region of Canada, near Edmonton, Alberta. The Canadian Entomologist 138: 376-383.

Mann, R.W. W.M. Bass, and L. Meadows. 1990. Time since death and decomposition of the human body: Variables and obsercations in case and experimental and field studies. Journal of Forensic Sciences 35: 103-111.

Payne, J.A. 1965. A summer carrion study of the baby pig *Sus scrofa* Linnaeus. Ecology 46: 592-602.

Payne, J.A. and E.W. King. 1972. Insect succession and decomposition of pig carcasses in water. Journal of the Georgia Entomological Society 7: 153-162.

Pujol-Luz, J.R. P. Abdon do Costa Francez, A. Ururahy-Rodrigues, and R. Constantino. 2008. The black soldier fly, Hermetia illucens (Diptera, Stratiomyodae), used to estimate the postmortem interval in a case in Amapá State, Brazil. Journal of Forensic Sciences 53: 476-478.

Reed, H.B. 1958. A study of dog carcass communities in Tennessee, with special reference to the insects. The American Midland Naturalist 59: 213-245.

Reibe, S. and B. Madea. 2010. How promptly do blowflies colonize fresh carcasses? A study comparing indoor with outdoor locations. Forensic Science International 195: 52-57.

Ross, A.H. S. M. Abel, and D. Radisch. 2009. Pattern of injury in child fatalities resulting from child abuse. Forensic Science International 188: 99-102.

Schoenly, K. M.L. Goff, J.D. Wells, and W.D. Lord. 1996. Quantifying statistical uncertainty in succession-based entomological estimates of the postmortem interval in death scene investigations: A simulation study. American Entomologist 42: 106-112.

Schoenly, K.G. N.H. Haskell, D.K. Mills, C. Bieme-Ndi, K. Larsen, andY. Lee. 2006. Recreating death's acre in the school yard: Using pig carcasses as model corpses to teach concepts of forensic entomology and ecological succession. The American Biology Teacher 68: 402-410.

Shahid, S.A. K. Schoenly, N.H. Haskell, R.D. Hall, and W. Zhang. 2003. Carcass enrichment does not alter decay rates or arthropod community structure: A test of the arthropod saturation hypothesis at the anthropology research facility in Knoxville, Tennessee. Journal of Medical Entomology 40: 559-569.

Tabor, K.L. C.C. Brewster, and R.D. Fell. 2004. Analysis of the successional patterns of insects on carrion in Southwest Virginia. Journal of Medical Entomology 41: 785-795.

Tabor, K.L. R.D. Fell, and C.C. Brewster. 2005. Insect fauna visiting carrion in Southwest Virginia. Forensic Science International 150: 73-80.

Tomberlin, J.K. and P.H. Adler. 1998. Seasonal colonization and decomposition of rat carrion in water and on land in an open field in South Carolina. Journal of Medical Entomology 35: 704-709.

Watson, E.J. and C.E. Carlton. 2003. Spring succession of necrophilous insects on wildlife carcasses in Louisiana. Journal of Medical Entomology 40: 338-347.

CHAPTER 2

Quantifying pteridines in the heads of blow flies: A novel application for forensic entomology

J.A. Cammack, A.C. Cohen, K.L. Kreitlow, R.M. Roe, and D.W. Watson

Key Words: *Chrysomya megacephala*, *Cochliomyia macellaria*, *Phormia regina*, spectrofluorometry

Abstract

In forensic cases involving entomological evidence, establishing the minimum postmortem interval (mPMI) is a critical component of the investigation that can help in case resolution. Traditional methods of estimating the mPMI rely on the aging of blow fly larvae collected from remains. However, if sufficient time has passed and the remains are in a late stage of decomposition, these insects have likely completed their development and may be present in the environment as adults. Unfortunately, adult fly collections are often ignored in cases of advance decomposition because of a presumed little relevance to mPMI. In this study we applied an under-used age-grading technique to age adults of *Ch. megacephala*, *C. macellaria*, and *P. regina* based on deposition of pteridines in the eyes. Fly age could be predicted with a high degree of accuracy (mean r^2 ± SE: 0.90 ± 0.01) for all species*sex*rearing temperature combinations except for males and females of *P. regina* reared at 5.4°C. Survival for all three species was highest at the low and intermediate temperatures and lowest at ~35°C, and *C. macellaria* had a higher rate of survival than did *Ch. megacephala* or *P. regina* at similar intermediate temperatures. These models can be used to increase the precision of entomology-based mPMI estimates for remains found during late decay, particularly those located indoors. The high r^2 of 22 of the 24 regression

equations indicates that this is a valid method for determining the age of adult blow flies at temperatures above 15°C.

Introduction

Establishing the minimum postmortem interval (mPMI) is a critical component of forensic investigations involving entomological evidence (Catts & Goff 1992). Traditional methods of estimating the mPMI rely on the aging of fly larvae collected from remains using time and temperature based developmental models. These models use laboratory-generated data on the development of larvae under controlled environmental conditions that approximate natural conditions for fly development. Most larval activity occurs during early and advanced decay, lasting approximately 14 days. Acts that result in the death of an individual are often concealed, thereby delaying discovery. Under such circumstances, immature fly stages may have completed development and thus occur as adults present in the indoor environment. Adult fly collections are often ignored in cases of advance decomposition.

The ecological process of decomposition is a complex series of cascading events involving abiotic and biotic factors that contribute to the reduction of biomass to its basal elements. Insects, particularly blow flies, often play a role in the decomposition process. Tomberlin et al. (2011) created a diagram of the decomposition process with respect to insect to insect activity in order to define and categorize events within this process (Figure 1).

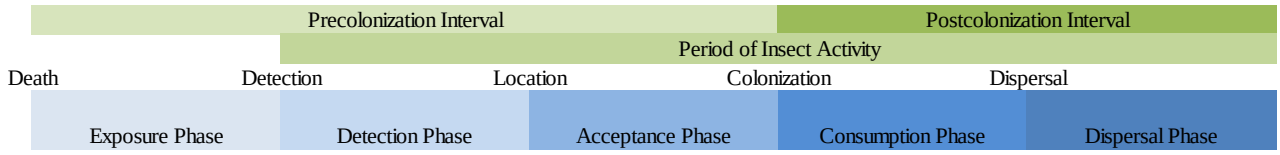


Figure 1. Stages of the vertebrate decomposition process, with respect to insect activity. Modified from Tomberlin et al. (2011).

Two specific intervals: the precolonization and postcolonization intervals (pre- and post-CI, respectively) make up the postmortem interval (PMI) (Tomberlin et al., 2011). Specifically, the colonization and consumption phase provide the most detail for mPMI estimates. Certain stimuli during the pre-CI attract adult flies to investigate the remains, and the resulting action is influenced by dietary needs, physiological age, and reproductive potential (Erzinçlioğlu, 1985; Goff 1991; Pujol-Luz et al., 2008). Arriving adult flies feed, mate, and/or oviposit on the remains. Knowledge of the age and reproductive status of these colonizing fly species would help to elucidate the mechanisms responsible for the duration of the pre-CI. In contrast, the post-CI has been widely studied, particularly in terms of larval development, which is used to estimate the age of immature insects collected from a corpse and provide an estimation of mPMI (Catts and Goff, 1992, Tarone & Foran 2011, Byrd & Allen 2001). In cases where remains are located indoors, or some other "sealed" location, such as a vehicle, the remains may be discovered after the initial colonizing larvae have completed their development and a large number of adults are present (the "Dispersal Phase" indicated in Figure 1). In such a situation, an entomological estimate of the mPMI is based on the entire duration of immature development and does not include the adults present at the scene, which are quite common (Benecke et al., 2004 and Parker et al., 2010). This omission

is a critical gap in the age determination of flies of forensic importance that would aid in estimating a more precise mPMI (Erzinçlioğlu 1986).

Occurrence of Remains Indoors

The discovery of decomposing remains located indoors that have been colonized by insects is quite high. In Germany over a 10 year period, entomological evidence was collected from 82% of 364 indoor cases (Frost et al. 2011). Similarly, in New Zealand, insects were collected in 74% of the 50 cases (Smeeton et al. 1984) and in 40% of 35 Hawaiian cases where the remains were located indoors (Goff 1991).

Goff (1991) compared differences in the insect species present on remains that were found indoors versus those found outdoors. Indoor species include the muscid *Musca domestica,* and the calliphorids *Chrysomya rufifacies* and *Ch. megacephala*. Dead pigs placed in indoor and outdoor locations were colonized by *Calliphora vicina* and *Lucilia sericata,* but *C. vicina* comprised 75% of all insects collected indoors, and only 15% of those collected outdoors (Reibe & Madea 2010). Similarly, we recovered nine blow fly species from pig carcasses indoors in Raleigh, NC and among them were *C. vicina*, *Ch. megacephala,* and *L. sericata* (Chapter 1).

Physiological Aging of Adult Blow Flies

Methods for aging adult flies have been developed, but they lack precision and have little application to forensic science (Zhu et al. 2013). Additionally, none of these methods/studies have been conducted on Nearctic dipteran populations. Data from Australia, and Central and South America may not apply to fly populations from North America. Techniques based on female reproductive physiology have been used for aging females of

higher Dipterans but are imprecise and require tedious dissections. Unfortunately, no methods for aging male flies based on reproductive physiology exist aside from separating mated from virgin males (Hayes & Wall 1999). This is problematic because many blow fly species exhibit protandry, a phenomenon where males emerge prior to females (Buck 2001), and some species, such as *Chrysomya rufifacies* lay single-sex egg batches (Baumgartner 1993), making it possible for only male flies to be collected at a scene. Therefore, a method of aging adult flies that is independent of reproductive status is needed.

Pteridines are compounds responsible for eye coloring in flies, and increase at a temperature-dependent rate as flies age. Pteridine accumulation curves have been generated using spectrofluorometry and have been used to successfully age blow flies. In *Chrysomya bezziana*, pteridine fluorescence was significantly correlated (mean r^2: 0.94, range: 0.90-0.98) with age and sex at 20, 25, and 30°C (Wall et al. 1990). *Lucilia sericata* exhibited similar multiplicative pteridine accumulation over time at 15, 20, 22.5, 27.5, and 32.5°C for both sexes; however, the r^2 values were lower for *L. sericata* (mean r^2: 0.88, range: 0.76-0.94) (Wall et al. 1991). In contrast to both *C. bezziana* and *L. sericata*, pteridines accumulate linearly in *Cochliomyia hominivorax* at 25 and 30°C, and could be used to predict age with about the same accuracy (r^2: 0.90) (Thomas & Chen 1989). Although variable between species, pteridines provide a reliable estimate of adult fly age, as long as size, sex, and the temperature under which the insect was reared are known. Recently, pteridine fluorescence was used to age grade male and female adult flesh flies, *Boettcherisca peregrina* reared at 5 temperatures for 15 to 24 days (Zhu et al. 2013). Their data illustrate a strong linear relationship between pteridine accumulation and male or female fly age

(r^2 = 0.882 to 0.958 and 0.910 to 0.957, respectively) (Zhu et al. 2013).

In this paper, we have applied techniques to age adult flies of forensic importance. Age grading adult flies can provide information about the population dynamics of blow flies that colonize remains, and the factors driving the colonization process. More importantly, age grading provides an accurate reproducible method of aging adult flies present at the scene beyond larval development. Therefore, the goal of this study is to apply methodology for aging three species of forensically-important blow flies commonly found colonizing remains located indoors: *Chrysomya megacephala*, *Cochliomyia macellaria*, and *Phormia regina*. These data can be us used to increase the precision of entomology-based mPMI estimates for remains found indoors.

Materials and Methods

Identification, Colony Establishment, and Rearing of Blow Fly Species

Three species of blow fly; *Chrysomya megacephala*, *Cochliomyia macellaria*, and *Phormia regina*, were selected for study because of their frequency of association with indoor porcine remains (Chapter 1). To establish colonies, wild flies were collected either as adults visiting decomposing remains or as larvae dispersing from decomposing remains (and identified post eclosion). Fly colonies were maintained at approximately 25.5°C and 70% RH, on a 16L:8D photoperiod, and were provisioned with water, granulated sucrose, and powdered milk, *ad libitum*. Three to five days prior to when eggs were needed, flies were provided with a rich protein source (fresh beef liver) to stimulate egg production (Rasso & Fraenkel 1954). Eggs were collected within 2 hrs of oviposition, transferred to a larval rearing medium, and allowed to complete development at 25.5°C and 70% RH, on a 16L:8D

photoperiod. For each species, adult flies were collected within 4 hrs of emergence from the puparia. Flies were separated into four groups of 17 age-discrete cohorts of a minimum of 10 individuals and reared in incubators (Fisher Scientific Isotemp BOD Refrigerated Incubators) on a 16:8 L:D photoperiod. Flies were provided with water and a mixture of granulated sugar and powdered milk. Rearing temperatures were chosen according to the temperatures at which each species is active in the wild. *Phormia regina*, a species which is active throughout much of the year, was reared at approximately 5, 15, 25, and 35°C. *Chrysomya megacephala* and *Cochliomyia macellaria*, species active during warmer portions of the year, were reared at approximately 15, 22, 28, and 35°C. The humidity within the rearing containers did not differ from that within the incubators, but did differ by temperature (Table 1). Cohorts of 10 flies were removed from the incubators at age = 0 d, 1 d, 2 d, and every 2 d until age = 30 d, and placed in a freezer (-20°C) until cold anesthetized. The flies were then transferred to vials, wrapped in foil, and stored protected from light at -20°C to protect pteridines from photo-deterioration. All subsequent processing occurred in a darkroom with a red light (wavelength ~ 675 nm) to prevent the degradation of pteridines through light exposure.

Table 1. Absolute humidity recorded within the incubators and rearing containers, at each of ten rearing temperatures.

Incubator Temperature °C	Absolute Humidity (g/m^3)
5.4	1.7
14.85	3.1
15.23	3.2
15.32	3.2
21.27	4.4
21.33	4.4
24.4	5.2
27.52	6.3
34.43	8.6
34.64	8.7

Quantifying Accumulation of Pteridines

Since pteridines are stored in the eyes of adult flies, the amount of pteridines present in the fly is positively correlated with body size. Therefore, pteridine fluorescence must be corrected for body size to standardize fluorescence values. Head width of each fly was measured to the nearest 0.5mm, using an ocular micrometer on a Nikon® SMZ-2T dissecting microscope. The flies were decapitated after head-width measurement, and the heads placed in Fisherbrand® 1.5 ml Snap-Cap® microcentrifuge tubes (Fisher Scientific, Waltham, MA, USA). To develop standard curves for pteridine fluorescence for each fly species, rearing temperature, and age, a modified version of the protocol of Lehane and Mail (1985) was used. Fly heads were homogenized with a pestle in centrifuge tubes containing 250 μl of 50 mM pH 8.0 tris-HCl buffer. An additional 750 μl of buffer was added, and each sample centrifuged at 6,000 rpm for 5 min. An aliquot (200 μl) of supernatant from each sample was transferred into one well of a black, polystyrene, 96 well microplate (Whatman Inc., Florham Park, NJ). Spectrofluorometry

was performed on a Molecular Devices FilterMax F5 Multi-Mode Microplate Reader (Molecular Devices, LLC, Sunnyvale, CA), with excitation set at 360 nm, emission at 450 nm, and integration at one second. Three standards (200 µl each) were run with each plate to ensure detector calibration and plate consistency: 1) 10 µg/ml pterin (Sigma-Aldrich, Inc., St. Louis, MD) in 1 M NaOH, 2) 50 mM tris-HCl buffer, and 3) distilled water. Relative fluorescence/ head capsule width was plotted against chronological age (hours) to develop formulae for predicting fly age at each rearing temperature.

All statistical analyses were conducted in SAS® v 9.3 (SAS® Institute, Cary, NC, USA). Accumulation rates of pteridines were compared across species, sex, and rearing temperatures, using PROC GLM. To select which regression model was the best at predicting fly age, linear and non-linear models for each species, sex, and rearing temperature were compared using Akaike information criteria (AIC) and Bayesian information criteria (BIC) maximum likelihood analyses. Regression equations were then generated using PROC REG.

Results

The rate of pteridine accumulation was positively correlated with and highly dependent on temperature for each species (Figures 2-4) (*Ch. megacephala*: $F_{3,505} = 60.67$, $p < 0.0001$; *C. macellaria*: $F_{3,525} = 46.08$, $p < 0.0001$; *P. regina*: $F_{3,502} = 81.83$, $p < 0.0001$). Females and males of all three species accumulated pteridines at different rates across all temperatures tested, except for *P. regina* at the low and high temperatures (Table 2), and each species accumulated pteridines at different rates when compared by sex (♀: $F_{2,782} = 7.79$, $p = 0.0004$; ♂: $F_{2,761} = 36.64$, $p < 0.0001$). The differential rate of accumulation is most

visible in *Ch. megacephala* at 34.64 and 26.26°C (Figure 2). The higher groups of data points at both temperatures represent the males, and the lower groups the females.

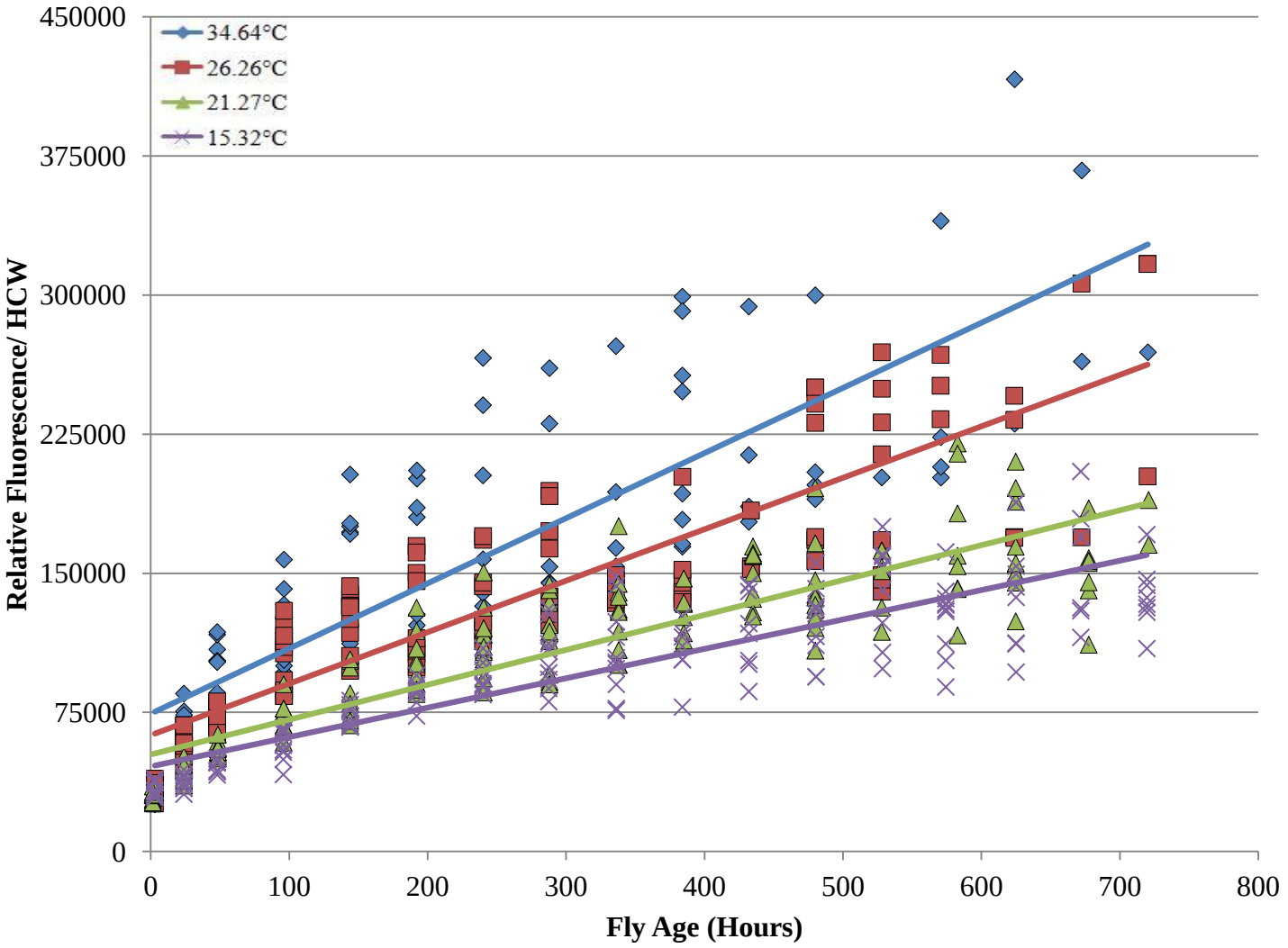


Figure 2. Accumulation of pteridines in adults of *Chrysomya megacephala* when reared at four different temperatures.

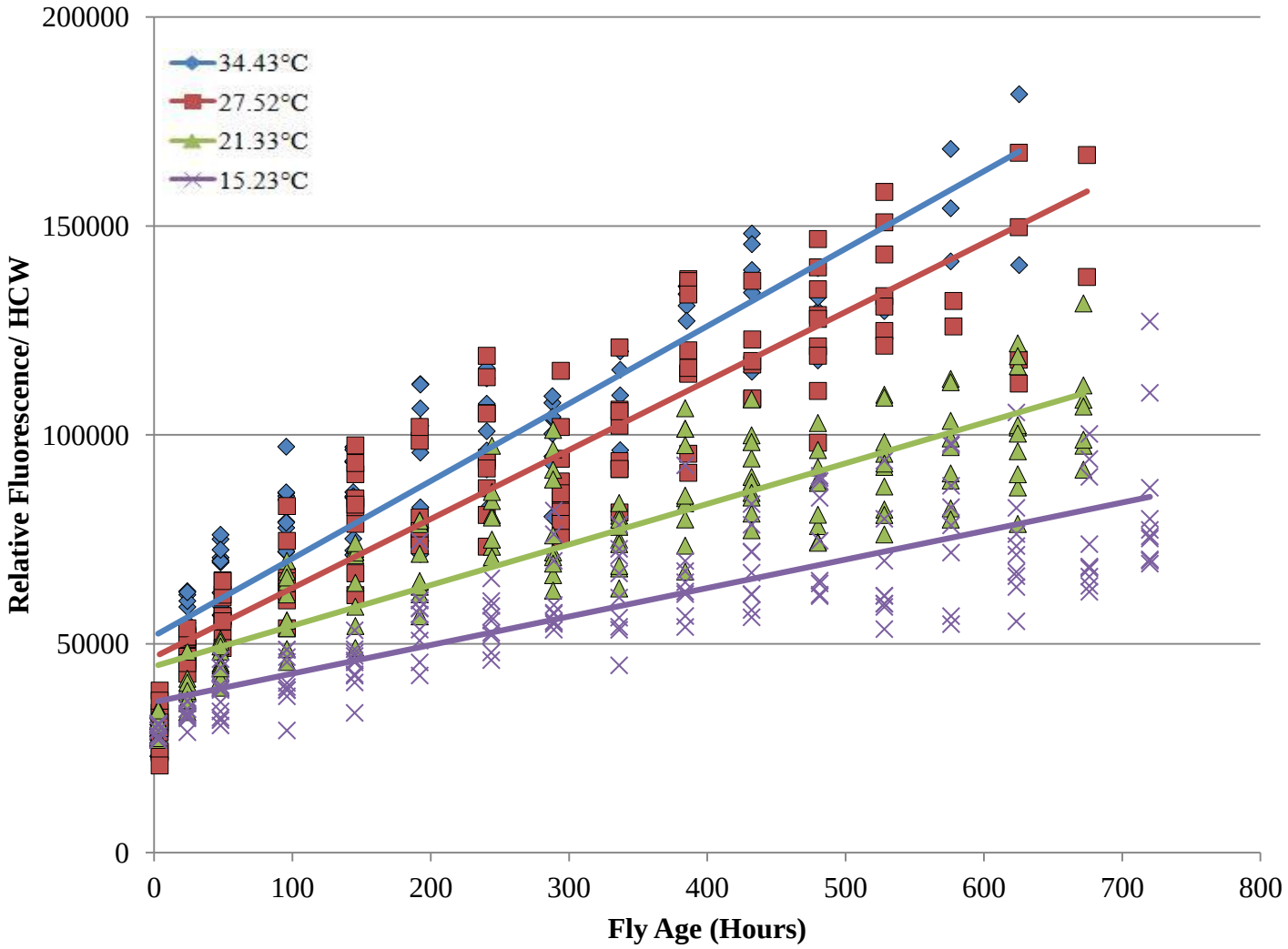


Figure 3. Accumulation of pteridines in adults of *Cochliomyia macellaria* when reared at four different temperatures.

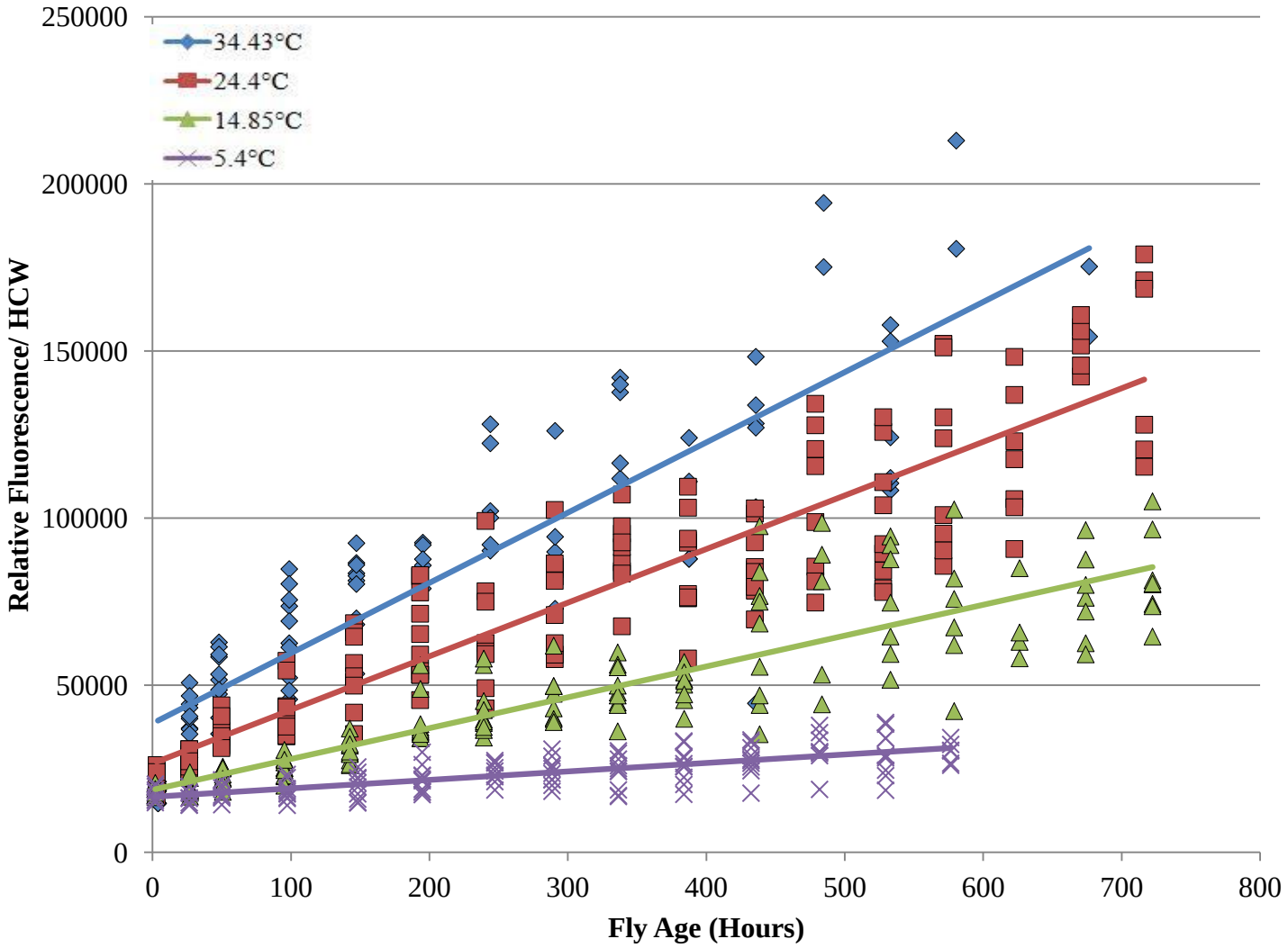


Figure 4. Accumulation of pteridines in adults of *Phormia regina* when reared at four different temperatures.

Table 2. Test statistics for accumulation rates of pteridines when compared by sex.

	Temperature	F-statistic	p-value
Ch. megacephala	15.32°C	$F_{1,152}$	<0.0001
	21.27°C	$F_{1,146}$	<0.0001
	26.26°C	$F_{1,108}$	<0.0001
	34.64°C	$F_{1,91}$	<0.0001
C. macellaria	15.23°C	$F_{1,155}$	<0.0001
	21.33°C	$F_{1,149}$	0.0011
	27.52°C	$F_{1,117}$	0.0002
	34.43°C	$F_{1,95}$	0.0037
P. regina	5.4°C	$F_{1,125}$	=0.11
	14.85°C	$F_{1,136}$	<0.0001
	24.4°C	$F_{1,146}$	<0.0001
	34.43°C	$F_{1,87}$	=0.25

Both AIC and BIC maximum likelihood analyses indicated that the same regression models for each species, sex, and rearing temperature combination were the most accurate models for predicting adult fly age (Table 3). The most accurate regression models were non-linear for all but males and females of *P. regina* reared at 5.4°C, and fly age could be predicted with a high degree of accuracy (mean R^2: 0.90 ± 0.01) for all species*sex*rearing temperature combinations for all but males and females of *P. regina* reared at 5.4°C (Table 3). Survival for all three species was highest at the low and intermediate temperatures and lowest at ~35°C, and *C. macellaria* had a higher rate of survival than did *Ch. megacephala* or *P. regina* at similar intermediate temperatures (Figure 5).

Table 3. Regression analyses of relative fluorescence/HCW against fly age (hours) for females and males of each species at different temperatures, where y = predicted age in hours and x = relative fluorescence of pteridines/HCW.

	Temperature (°C)	Sex	Regression Model	R^2	n
Ch. megacephala	15.32	F	$y = 1.76E^{-7}x^2 - 6.86E^{-13}x^3 + 87.04$	0.85	74
		M	$y = 7.97E\text{-}8x^2 - 2.49E\text{-}13x^3 + 21.45$	0.86	82
	21.27	F	$y = -0.0059x + 1.11E^{-7}x^2 - 3.28E^{-13}x^3 + 91.40$	0.89	75
		M	$y = -0.0077x + 1.047E^{-7}x^2 - 2.77E^{-13}x^3 + 176.37$	0.84	75
	26.26	F	$y = -0.0059x + 7.97E^{-8}x^2 - 1.83E^{-13}x^3 + 122.28$	0.90	52
		M	$y = -0.0025x + 3.04E^{-8}x^2 - 5.07E^{-14}x^3 + 68.08$	0.97	60
	34.64	F	$y = -0.0023x + 3.72E^{-8}x^2 - 7.22E^{-14}x^3 + 39.52$	0.96	52
		M	$y = -0.00097x + 1.19E^{-8}x^2 - 1.39E^{-14}x^3 + 29.13$	0.96	43
C. macellaria	15.23	F	$y = -0.071x + 1.66E^{-6}x^2 - 1.012E^{-11}x^3 + 939$	0.84	90
		M	$y = -0.0092x + 2.78E^{-7}x^2 - 1.30E^{-12}x^3 + 73.47$	0.88	69
	21.33	F	$y = -0.027x + 5.54E^{-7}x^2 - 2.62E^{-12}x^3 + 388.92$	0.87	82
		M	$y = -0.024x + 3.80E^{-7}x^2 - 1.40E^{-12}x^3 + 447.05$	0.88	71
	27.52	F	$y = -0.010x + 2.11E^{-7}x^2 - 8.10E^{-13}x^3 + 127.69$	0.91	43
		M	$y = -0.0075x + 1.26E^{-7}x^2 - 3.85E^{-13}x^3 + 126.69$	0.92	78
	34.43	F	$y = -0.0055x + 1.14E^{-7}x^2 - 3.55E^{-13}x^3 + 69.98$	0.92	41
		M	$y = -0.0079x + 1.16E^{-7}x^2 - 3.16E^{-13}x^3 + 141.51$	0.96	58
P. regina	5.4	F	$y = -0.037x + 155.39$	0.53	71
		M	$y = -0.061x + 403.48$	0.57	58
	14.85	F	$y = 3.40E^{-7}x^2 - 2.67E^{-12}x^3 - 67.81$	0.91	93
		M	$y = 2.82E^{-7}x^2 - 1.68E^{-12}x^3 + 47.41$	0.88	47
	24.4	F	$y = 1.07E^{-7}x^2 - 5.35E^{-13}x^3 - 70.37$	0.94	71
		M	$y = 7.99E^{-8}x^2 - 2.83E^{-13}x^3 - 3.10$	0.95	79
	34.43	F	$y = 1.13E^{-7}x^2 - 4.54E^{-13}x^3 + 40.97$	0.80	44
		M	$y = -0.0038x + 7.81E^{-8}x^2 - 2.32E^{-13}x^3 + 78.54$	0.81	47

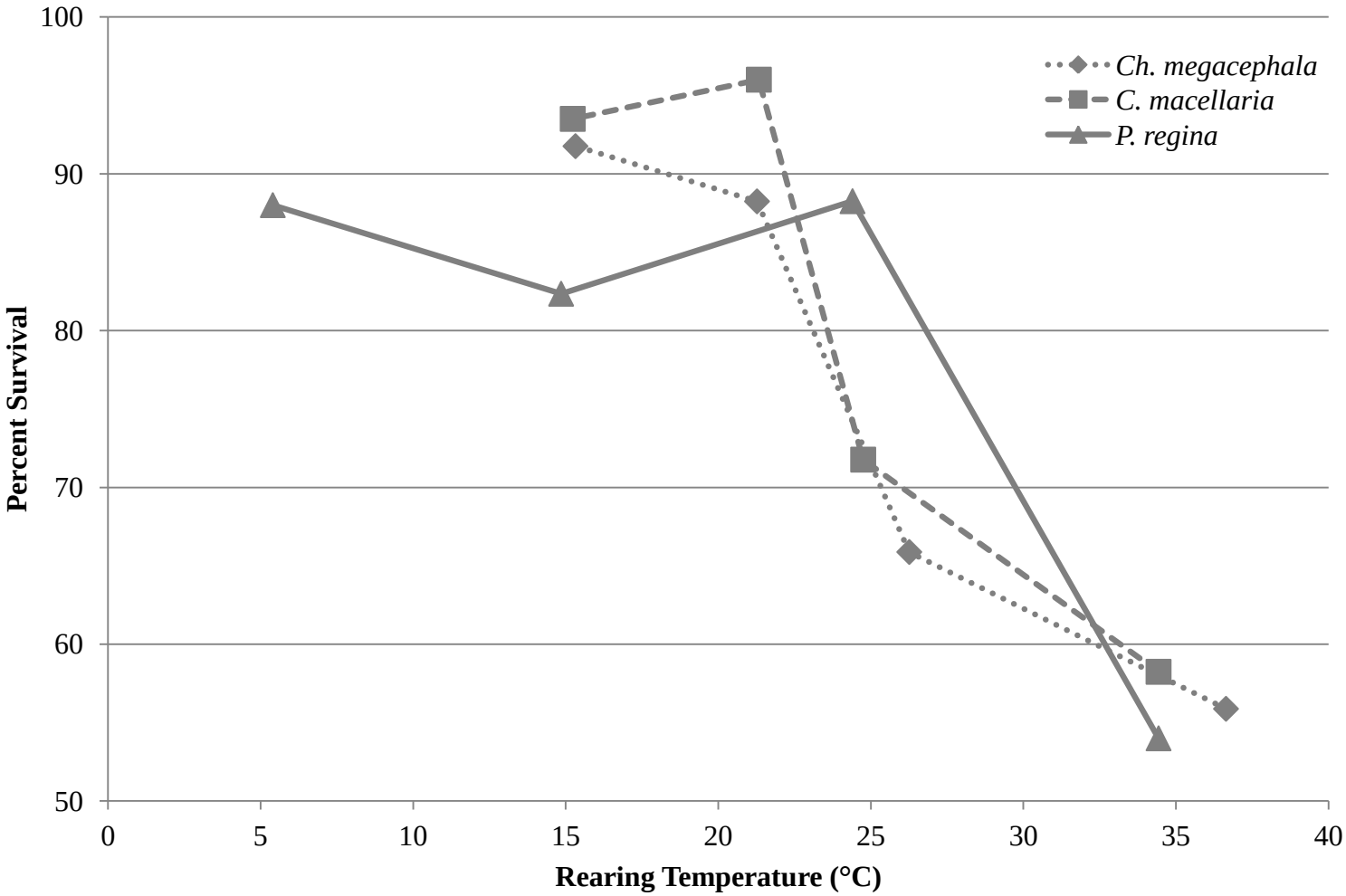


Figure 5. Percentage of adults surviving at each rearing temperature for each of the three species studied.

Discussion

Quantifying pteridines in the heads of *Ch. megacephala*, *C. macellaria*, and *P. regina* is an effective method for determining the age of adult flies. The age of flies can be predicted with greater than 80% accuracy at temperatures above 15°C. Being able to age adult flies collected at body-recovery scenes when the remains are in a late stage of decomposition will increase the accuracy of entomology-based estimates of the mPMI in cases involving remains located indoors. This study represents the first of its kind to quantify pteridines in the heads of blow flies for forensic purposes, and is the first to develop pteridine accumulation curves for Nearctic populations of blow flies.

In contrast to previous studies on cyclorrhaphan dipterans (e.g. Mail et al., 1983; Thomas & Chen 1989; Zhu et al., 2003, 2013) that found pteridines to accumulate linearly with fly age, in our study, maximum likelihood analyses indicated that non-linear models were the most accurate for predicting fly age for all but adults of *P. regina* reared at 5.4°C. Zhu et al. (2013) evaluated non-linear models, but the data were not presented and their methodology for rejecting the non-linear models in favor of linear models is unknown. Pteridines accumulated linearly in adults of *Ch. megacephala* from China (Zhu et al., 2003), suggesting that populations of *Ch. megacephala* differ between China and North Carolina. No prior studies have quantified pteridines in Nearctic populations of blow flies, and the differences we found between our *Ch. megacephala* and those from China suggest that models developed on populations of other species in Australia (Wall et al., 1990, 1991), China (Zhu et al., 2003, 2013), and Central America (Thomas & Chen 1989, 1990) might not accurately predict the age of forensically-important flies in other regions of the world.

Although statistical analyses indicated that females and males of *P. regina* did not accumulate pteridines at different rates when reared at 5.4 or 34.43°C (see Table 2), separate regression models for each sex and rearing temperature are included in Table 2, because all prior studies quantifying pteridines in blow flies indicate that accumulation rates differ by sex (e.g. Wall et al., 1990, 1991; Zhu et al., 2003). The low r^2 and relatively flat slope of the models developed for *P. regina* at 5.4°C can be attributed to the lack of activity when reared at that temperature; pteridines are a by-product of purine metabolism, and metabolic activity of the flies was low as indicated by their lack of physical activity and low consumption of food and water. Although adults of *P. regina* are present in North Carolina and much of the U.S. during the spring and fall when ambient temperatures are typically that low, they are probably able to thermoregulate via sun exposure to increase activity. The low survival of *Ch. megacephala* and *C. macellaria* when compared to *P. regina* at temperatures above 25°C suggests that Raleigh, NC populations of these two species might not be as heat tolerant as suggest in published bionomic accounts (Abdul-Rassoul et al., 2009; Hall 1947). Perhaps like the invasive *Ch. rufifacies* (Cammack & Nelder 2010), invasive populations of *Ch. megacephala* might be acclimatizing to cooler conditions.

Pteridines can be extracted from the heads of flies stored dry at room temperature for up to 8 weeks (Perez-Mendoza et al., 2002; no comparative data presented) as long as they are stored protected from light. This should allow sufficient time following collection at a scene for specimens to be transferred to the proper laboratory for analysis. Ideally, flies should be collected into vials or other small containers, the containers wrapped in foil to protect the specimens from light and prevent degradation of pteridines, and the flies killed by

freezing. Specimens should be kept frozen until they can be transferred to the proper laboratory for analysis.

The methodology used in the current study quantifies all pteridines in the heads of blow flies. The pteridines present in the heads of a number of species of dipterans have been identified (Mail & Lehane 1988), and different pteridines accumulate differently as adults of *Stomoxys calcitrans* (L.) (Mail & Lehane 1988) and *Anastrepha ludens* (Loew) (Tomic-Carruthers et al., 2002) age. Future work on quantifying pteridines should investigate the change in the ratios of these different pteridines as flies age, or quantify individual pteridines, as a way to increase the precision and accuracy of aging flies based on pteridine accumulation. The application of the developed models also should be tested on regional fly populations to determine the range of validity and application to forensic casework.

Acknowledgement

We thank S.S. Denning and L.M. Guisewite for their assistance with sample collection and M.H. Reiskind for assistance with statistical analyses. This work was partially funded by NIJ Grant 1864 2011-0002.

References

Abdul-Rassoul, M.S. R.S.H. Augul, and H. Al-Saffar. 2009. 2009. Seasonal abundance of third instar larvae of flies (Order: Diptera) on the exposed carcasses. Bulletin of the Iraqi National History Museum 10: 1-9.

Baumgartner, D.L. 1993. Review of *Chrysomya rufifacies* (Diptera: Calliphoridae). Journal of Medical Entomology 30: 338-352.

Benecke M., E. Josepji, & R. Zweihoff. 2004. Neglect of the elderly: Forensic entomology cases and considerations. Forensic Science International 146S: S195-S199.

Buck, M. 2001. Protogyny, protandry, and bimodal emergence patterns in necrophagous Diptera. The Canadian Entomologist 133: 521-531.

Byrd, J.H. and J.C. Allen. 2001. The development of the black blow fly, *Phormia regina* (Meigen). Forensic Science International 72: 79-88.

Cammack, J.A. and M.P. Nelder. 2010. Cool-weather activity of the forensically important hairy maggot blow fly *Chrysomya rufifacies* (Macquart) (Diptera: Calliphoridae) on carrion in Upstate South Carolina, United States. Forensic Science International 195: 139-142.

Catts, E.P. and M.L. Goff. 1992. Forensic entomology in criminal investigations. Annual review of Entomology 37: 253-272.

Erzinçlioğlu, Y.Z. 1985. The entomological investigation of a concealed corpse. Med Sci Law 25: 228-230.

Erzinçlioğlu, Y.Z. 1986. Areas of research in forensic entomology. Medicine, Science, and the Law 26: 273-278.

Frost, C.L. H.R. Braig, J. Amendt, and M.A. Perotti. 2011. Indoor arthropods of forensic importance: Insects associated with indoor decomposition and mites as indoor markers. In *Current Concepts in Forensic Entomology*, J. Amendt, M.L. Goff, C.P. Campobasso, and M. Grassberger, eds. Springer, Dordrecht, Netherlands. Pp 93-108.

Goff, M.L. 1991. Comparison of insect species associated with decomposing remains recovered inside dwellings and outdoors on the island of Oahu, Hawaii. Journal of Forensic Sciences 36: 748-753.

Hall, D.G. 1947. *The Blowflies of North America*. The Thomas Say Foundation. 475 Pp.

Hayes, E.J. and R. Wall. 1999. Age-grading adult insects: A review of techniques. Physiological Entomology 24: 1-10.

Mail, T.S. J. Chadwick, and M.J. Lehane. 1983. Determining the age of adults of *Stomoxys calcitrans* (L.) (Diptera: Muscidae). Bulletin of Entomological Research 73: 501-525.

Mail, T.S. and M.J. Lehane. 1988. Characterisation of pigments in the head capsule of the adult stablefly *Stomoxys calcitrans*. Entomologia Experimentalis et Applicata 46: 125-131.

Parker, M.A. M. Benecke, J.H. Byrd, R. Hawkes, and R. Brown. 2010. Entomological alteration of bloodstain evidence. In *Forensic Entomology: The Utility of Arthropods in Legal Investigations*, J.H. Byrd and J.L. Castner, eds. CRC Press, Boca Raton, Florida, USA. Pp 539-380.

Perez-Mendoza, J. F.E. Dowell, A.B. Broce, J.E. Throne, R.A. Wirtz, F. Xie, J.A. Fabrick, and J.E. Baker. 2002. Chronological age-grading of house flies by using near-infrared spectroscopy. Journal of Medical Entomology 39: 499-508.

Pujol-Luz J.R. P.A.D.C. Francez, A. Ururahy-Rodrigues, and R. Constantino. 2008. The black soldier-fly, *Hermetia illucens* (Diptera, Stratiomyidae), used to estimate the postmortem interval in a case in Amapa State, Brazil. Journal of Forensic Sciences 53: 476-478.

Rasso, S.C. and G. Fraenkel. 1954. The food requirements of the adult female blow-fly, *Phormia regina* (Meigen), in relation to ovarian development. Annals of the Entomological Society of America 47: 636-654.

Smeeton, W.M.I. T.D. Koelmeyer, B.A. Holloway, and P. Singh. 1984. Insects associated with exposed human corpses in Auckland, New Zealand. Medicine, Science, and the Law 24: 167-174.

Tarone, A.M. and D.R. Foran. 2011. Gene expression during blow fly development" Improving the precision of age estimates in forensic entomology. Journal of Forensic Sciences 56: S112-S122.

Thomas, D.B. and A.C. Chen. 1989. Age determination in the adult screwworm (Diptera: Calliphoridae) by pteridine levels. Journal of Economic Entomology 82: 1140-1144.

Thomas, D.B. and A.C. Chen. 1990. Age distribution of adult female screwworms (Diptera: Calliphoridae) captured on sentinel animals in the coastal lowlands of Guatemala. Journal of Economic Entomology 83: 1422-1429.

Tomberlin, J.K. R. Mohr, M.E. Benbow, A.M. Tarone, and S. VanLaerhoven. 2011. A roadmap for bridging basic and applied research in forensic entomology. Annual Review of Entomology 56: 401-421.

Tomic-Carruthers N. R. Mangan, and R. Carruthers. 2002. Age estimation of Mexican fruit fly (Diptera: Tephritidae) based on accumulation of pterins. Journal of Economic Entomology 95: 1319-1325.

Wall, R. P.A. Langley, J. Stevens, and G.M. Clarke. 1990. Age-determination in the old-world screw-worm fly *Chrysomya bezziana* by pteridine fluorescence. Journal of Insect Physiology 36: 213-218.

Wall, R. P.A. Langley, & K.L. Morgan. 1991. Ovarian development and pteridine accumulation

for age determination in the blowfly *Lucilia sericata*. Journal of Insect Physiology 37: 863-868.

Zhu, G.H. G.Y. Ye, and C. Hu. 2003. 2003. Determining the adult age of the oriental latrine fly, *Chrysomya megacephala* (Fabricius) (Diptera: Calliphoridae) by pteridine fluorescence analysis. Entomologia Sinica 10: 245-255.

Zhu, G.H. G.Y. Ye, K. Li, C. Hu, & X.H. Xu. 2013. Determining the age of adult flesh flies , *Boettcherisca peregrina,* using pteridine fluorescence. Medical and Veterinary Entomology 27: 59-63.

CHAPTER 3

Effects of duration and delay of on-site temperature recording on retrospective temperature estimation: Implications for forensic entomology

J.A. Cammack[1], L.M. Guisewite[1], P. Bloomfield[2], and D.W. Watson[1]

[1]Department of Entomology, North Carolina State University, Raleigh, NC, USA 27695
[2]Department of Statistics, North Carolina State University, Raleigh, NC, USA 27695

Abstract

Accurate temperature data from a body-recovery scene are required for the reliable analysis of entomological evidence collected from decomposing remains. Forensic entomologists typically collect temperature data on site and use regression analyses to predict on-site temperatures during the time prior to discovery of the remains and subsequent collection of entomological evidence. In this study, we investigated how the amount of time elapsed between discovery of remains and temperature data collection, as well as the duration of data collection affects the accuracy of these regression analyses. We found that as the amount of time between discovery and data collection increases, longer durations of data collection are necessary to produce accurate regression models. Ideally, data collection should begin as soon after discovery as possible, but accurate regression models can still be obtained when data collection begins up to 2 months after discovery of the remains.

Introduction

The application of forensic entomology relies on the ability to accurately identify the species and estimate the age the insects, particularly blow fly larvae, collected from decomposing remains (Catts and Goff, 1992), and the estimation of this age can be used to infer a minimum postmortem intermal (mPMI) of the decedent (Wells and Lamotte, 2010).

Although many factors influence the rate of development of forensically-important insects (Tarone and Foran, 2006), temperature is the most important. Since the rate of development of fly larvae is temperature dependent, determining the age of fly larvae is dependent on knowing the local temperatures under which those insects developed (Catts, 1992). Weather stations serve as a source of climate and temperature-related data for most investigations. However, forensic specialists recognize that there can be significant ecological and climatological differences between a body-recovery site and nearby weather stations (Scala and Wallace, 2010). Therefore, temperatures at the body-recovery site must be estimated over the time during which the remains lay *in situ*. This is done by collecting temperature data on site and regressing these data against data collected from the nearest weather station during the same time period; the resulting equation is then used to predict on–site temperatures during the period of time when the remains were *in situ* (Archer 2004). The time elapsed between discovery of the remains and the initiation of post-discovery data collection is largely dependent on when the entomologist is notified of the case. Inconsistencies in the recommended duration for data collection and frequency intervals vary from days to weeks. Amendt et al. (2006) recommend recording hourly data for 5-10 days, while Gennard (2007) and Anderson (2005) recommend recording temperatures for 3-5 days and 2-3 weeks, respectively, but neither specifies the frequency intervals. Haskell and Williams (2008) and Byrd et al. (2010) both recommend taking 3-4 readings per day for 3-4 days, but these recommendations are minimal and were likely developed for law enforcement agencies or crime labs without access to remote temperature data loggers. However, none of the aforementioned examples validate their recommendations. Two studies (Archer, 2004;

Johnson et al., 2012) have addressed the lack of empirical data and examined if this technique employed by forensic entomologists is in fact valid. Archer (2004) tested the accuracy of predicting 7 days' worth of temperature data (the hypothetical body *in situ* period), when placing a data logger on the site 1, 11, 29, and 78 days after discovery, for a duration of 10 days at each placement date. The predictive models using on site hourly temperature data were more accurate than weather station data in 22 of 24 of their correlations; weather station data were only more accurate than the predictive models at 78 days post discovery. Similarly, Johnson et al. (2012) tested the accuracy of predicting the previous 5 days of temperature data, by placing a data logger on site for 2, 5, or 10 days, post discovery of the remains. The predictive models based on temperature data collected on site were more accurate than weather station data in 92 of 96 of the correlations. From these studies it is clear that regression models developed from on-site temperature data collection post-discovery remains more accurately predict the *in situ* temperatures than those of the weather station data alone. However, we do not know the relative precision of that estimate relative to the time elapsed post-discovery.

The goal of the current study was to expand on that of the two previous studies, by determining the minimum duration for placement of data loggers that yields the most accurate predictive model possible. We hypothesized that as the length of time between discovery and data logger placement increased, so too would the amount of data collection needed to produce an accurate model.

Materials and Methods

Hobo® H8 relative humidity and temperature data loggers (Onset Computer Corporation, Bourne, MA, USA) were placed at three simulated body-recovery sites on the Poultry Field Laboratory at the North Carolina State University Lake Wheeler Road Field Laboratory, Raleigh, NC, USA. The sites were an open field, a deciduous forest, and inside an open barn. Loggers were programmed to record temperatures each hour, for approximately 150 days. All loggers were shaded to prevent direct sun exposure from increasing the recorded temperature, but an external temperature probe was also used on the logger placed in the open field to collect temperatures in direct sunlight. Hourly observed temperatures (measured on the hour) and hourly average temperatures (measured each minute and averaged) for the same 150 day period were obtained from the State Climate Office of North Carolina from a nearby weather station, located approximately 1 km north east of the study site. Logger and weather station data were paired by corresponding dates, and the data set split into six replicates. For each replicate, the first 10 days was selected as the hypothetical body *in situ* period, and did not overlap among replicates. Within each replicate, data were selected to simulate placing the data logger on-site at 4 different times (0, 20, 40, and 60 days post-discovery) and for 7 durations (1, 3, 5, 7, 10, 15, and 20 days) within each placement time (Figure 1).

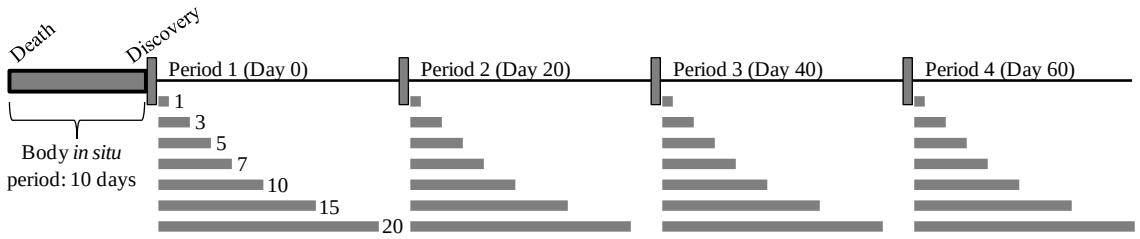


Figure 1. Diagram illustrating one replicate of the study. Each replicate was split into pre- (the body *in situ* period) and post-discovery (temperature data collection) phases. Post-discovery periods 1-4 were separated by 20 days each, and within each period temperatures were sampled for seven different durations and used to generate models to predict temperatures during the pre-discovery phase.

Time and duration of placement were regressed against weather station data to generate equations that could be used to predict site temperatures during the body *in situ* period. The absolute difference (residual) between predicted and actual temperatures at each site were calculated and analyzed by ANOVA followed by Tukey's Honest Significant Difference Test, using JMP Pro 9.0 (SAS Institute, Cary, North Carolina, USA).

Once the minimum required duration of logger placement for a linear model was determined, we compared the accuracy of temperatures predicted by a lagged regression model (using the corresponding, and one previous and subsequent hour as predictors) to those predicted by our linear regression models. The mean absolute differences between the on-site and predicted temperatures were taken for each model type (linear and lagged), and these were analyzed by ANOVA followed by Tukey's Honest Significant Difference Test, using JMP Pro 9.0 (SAS Institute, Cary, North Carolina, USA).

Field data were used to validate the resulting models and proposed methodology. Blow fly larvae were collected from a decomposing pig placed at the open field site. These

larvae were identified, and their age used to provide an estimate of the post-colonization interval.

Results

Linear Regression Models

Regression analyses generated 1,344 linear regression equations with an average R^2 of 0.83 ± 0.004. Hourly observed and average temperature data from the weather station were not significantly different ($F_{1, 13,438} = 0.59$, $p = 0.44$); therefore, hourly observed temperatures were used to maintain continuity with data collected by the data loggers. For all periods of placement, as the duration of logger placement increased, the mean absolute residual decreased (Period 1: $F_{6, 40,313} = 34.55$, $p < 0.0001$; Period 2: $F_{6, 40,313} = 31.7$, $p < 0.0001$; Period 3: $F_{6, 40,313} = 62.27$, $p < 0.0001$; Period 4: $F_{6, 40,313} = 77.18$, $p < 0.0001$) (Figure 2). Within each period, the duration of placement at which the residuals no longer differed was selected as the minimum amount of time a data logger would need to be left at a body-recovery scene to produce the most accurate predictive model. For period 1 (logger placement on the date of discovery), this corresponds to recording hourly temperatures for at least 5 days before the residuals were not significantly different (Figure 2, Period 1). For periods 2, 3 and 4 (logger placement 20, 40, or 60 days post discovery, respectively), temperature data were not significantly different after 10 days.

For all periods of logger placement, the temperatures estimated by the linear model from the predicted minimum duration of placement were a better estimate of on-site temperatures than were weather station data (Period 1: $F_{7, 46,072} = 32.91$, $p < 0.0001$; Period 2: $F_{7, 46,072} = 27.9$, $p < 0.0001$; Period 3: $F_{7, 46,072} = 56.49$, $p < 0.0001$; Period 4: $F_{7, 46,073} = 67.11$,

$p < 0.0001$) (Figure 3).

Lagged Regression Models

For each period of placement, lagged regression models produced significantly lower mean absolute residuals for only the data logger placed at the open field site (Period 1: $F_{5, 8634} = 469.62$, $p < 0.001$; Period 2: $F_{5, 8,634} = 339.66$, $p < 0.001$; Period 3: $F_{5, 8,634} = 466.92$, $p < 0.001$; Period 4: $F_{5, 8,634} = 685.13$, $p < 0.001$).

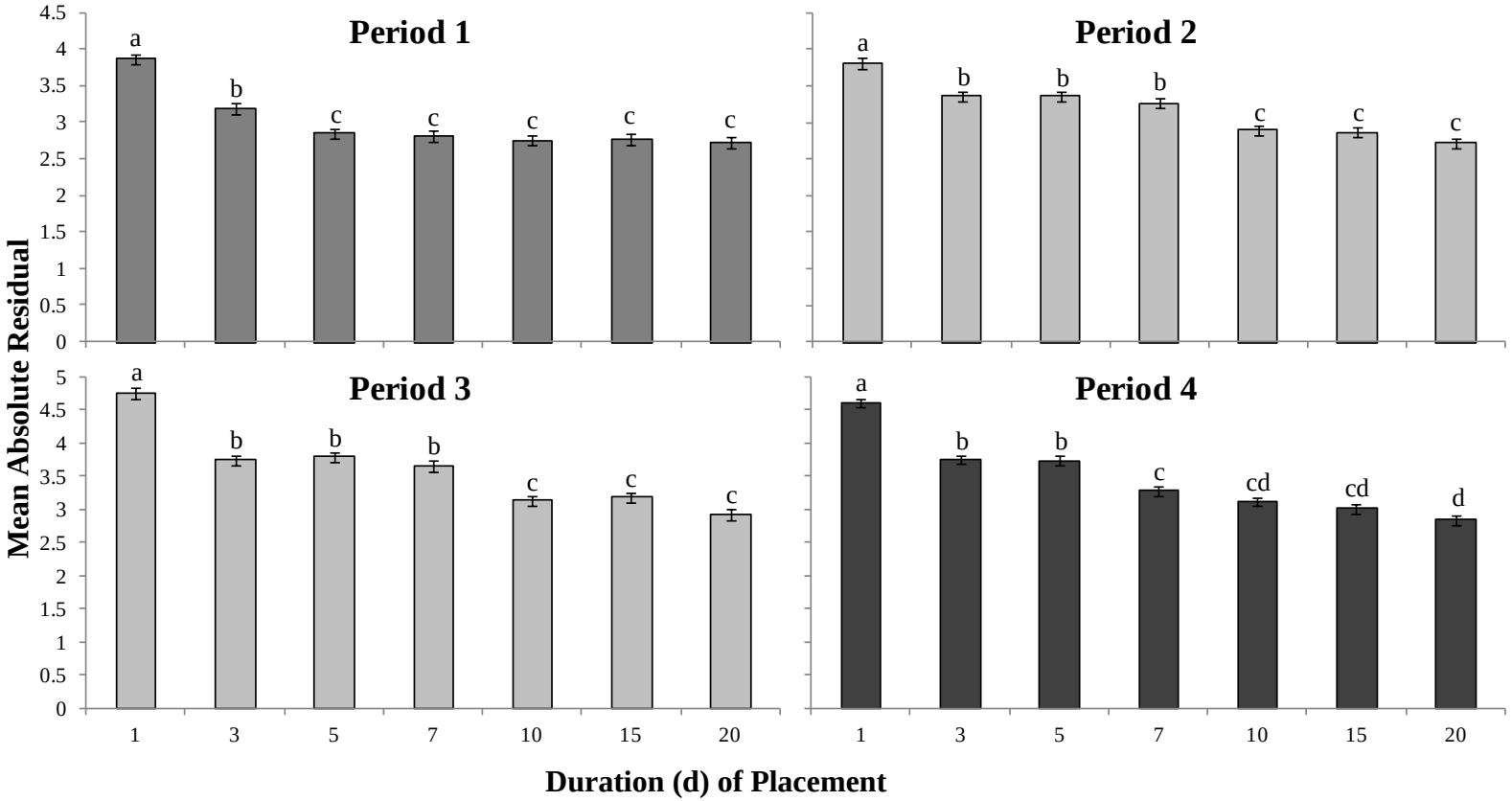


Figure 2: Mean absolute residuals across all sites, for each period and duration of placement tested. Different letters within a period indicate significant differences (α=0.05, Tukey's Honest Significant Difference Test).

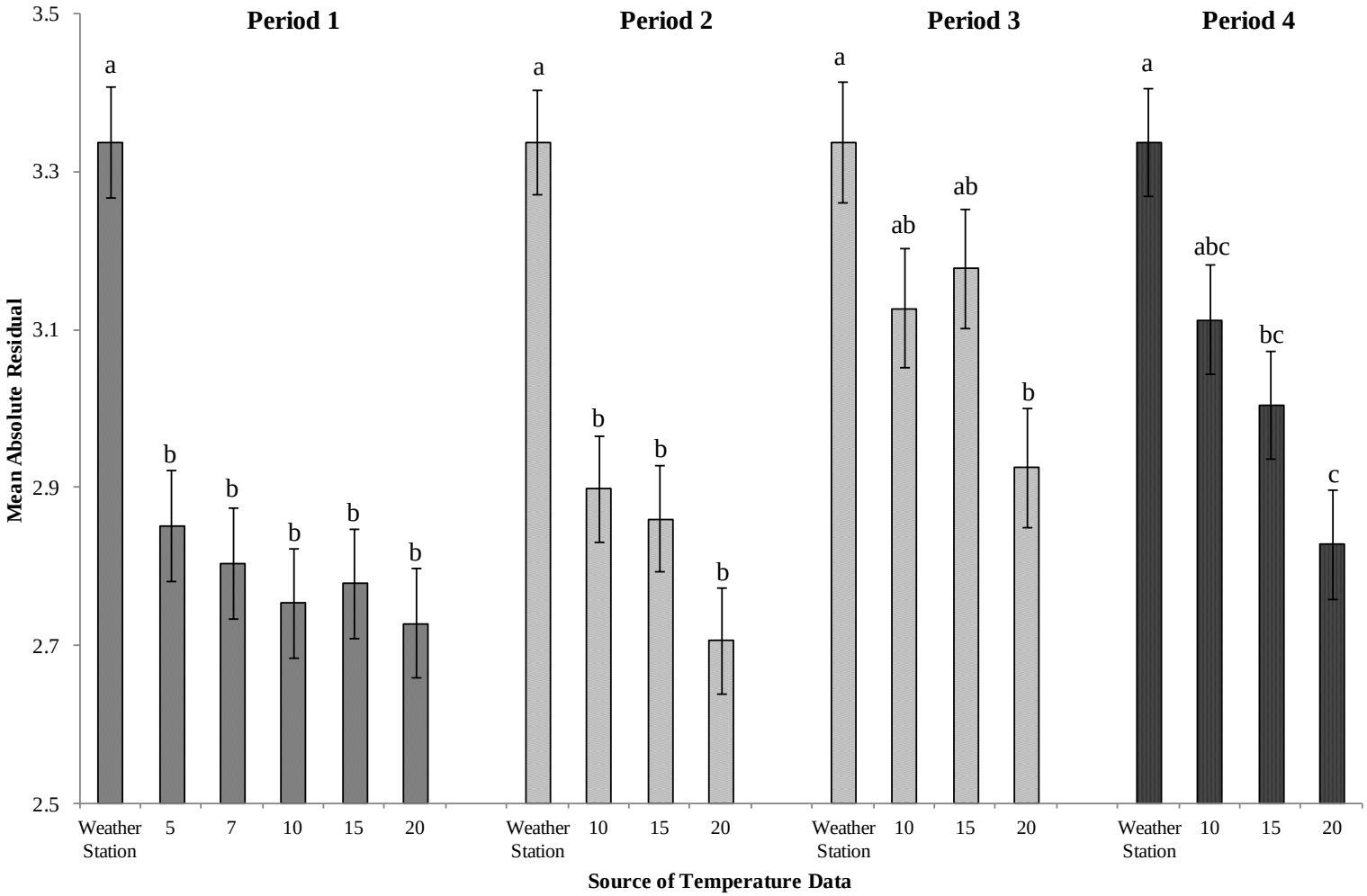


Figure 3. Comparison of the accuracy of weather station data to that predicted by linear regression models across all sites, for each period of logger placement. Different letters within a period indicate significant differences (α=0.05, Tukey's Honest Significant Difference Test).

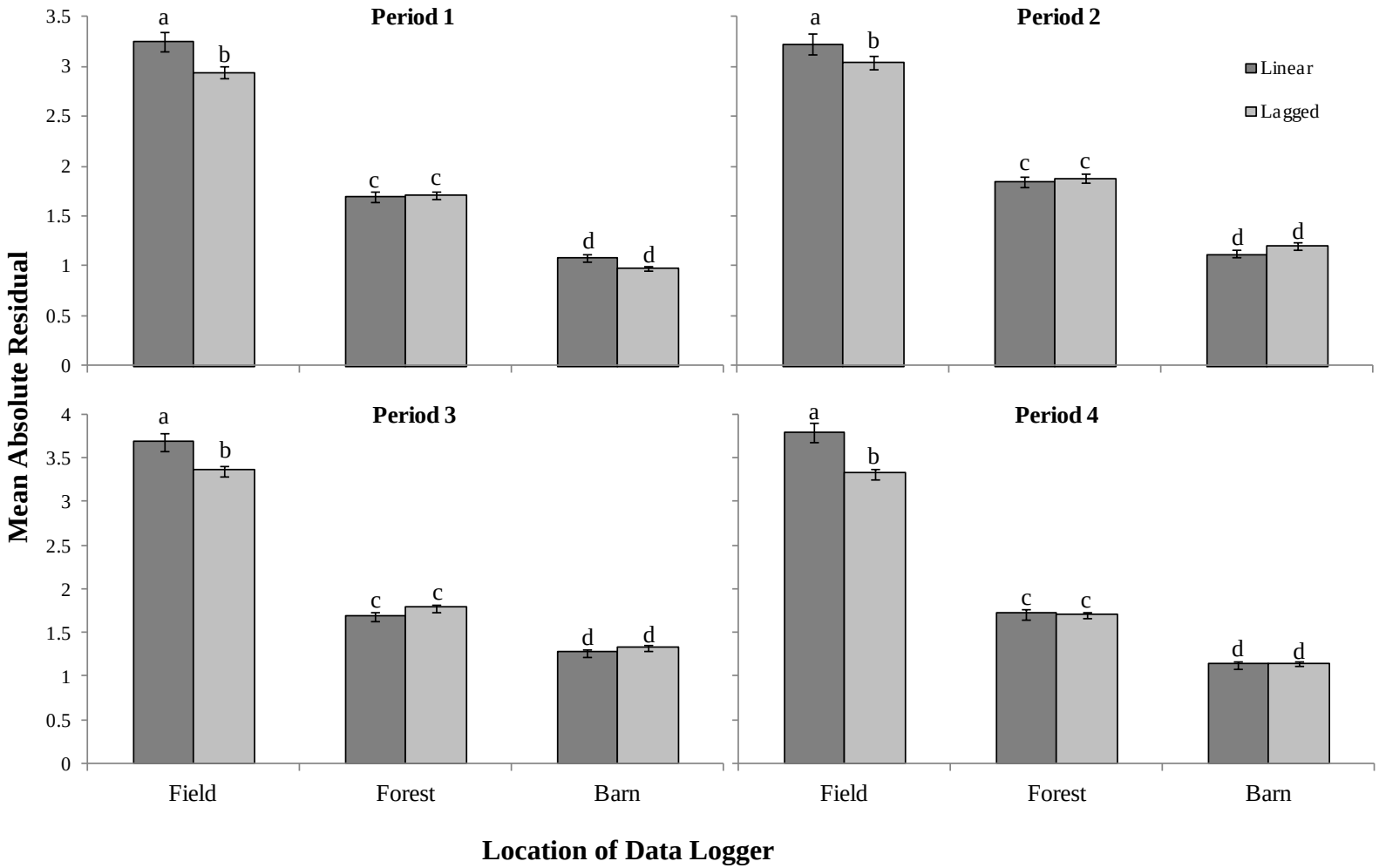


Figure 4. Comparison of mean absolute residuals from linear and lagged regression models, when data were selected to simulate logger placement for the minimum duration predicted by linear models. Different letters within a period of placement indicate significant differences (α=0.05, Tukey's Honest Significant Difference Test).

Validation Case Study

Larvae of *Phormia regina* Meigen were collected as third instars beginning to disperse from a pig carcass at the open field site, on 27 October 2010. The age of the larvae was calculated using the data set of Byrd and Allen (2001), which previous studies have shown to be the most accurate data set for *P. regina* in Raleigh, NC (Cammack, unpublished data), using a development threshold of 10°C (Higley and Haskell, 2000). Larvae of *P. regina* require a minimum of 1360 to 1530 accumulated degree hours to enter prepupal/dispersing stage, when reared at 15-20°C (Byrd & Allen, 2001). Temperature data were selected to simulate logger placement on 27 October 2010 for a duration of 5 days, and 20 days later for a duration of 10 days. On-site temperatures were then predicted using linear and lagged regression models. Although these estimates predicted that colonization occurred 23-47 hours after eggs were first present, they were more accurate than using weather station data alone, which predicted that colonization occurred 69 hours after eggs were first present (Figure 5).

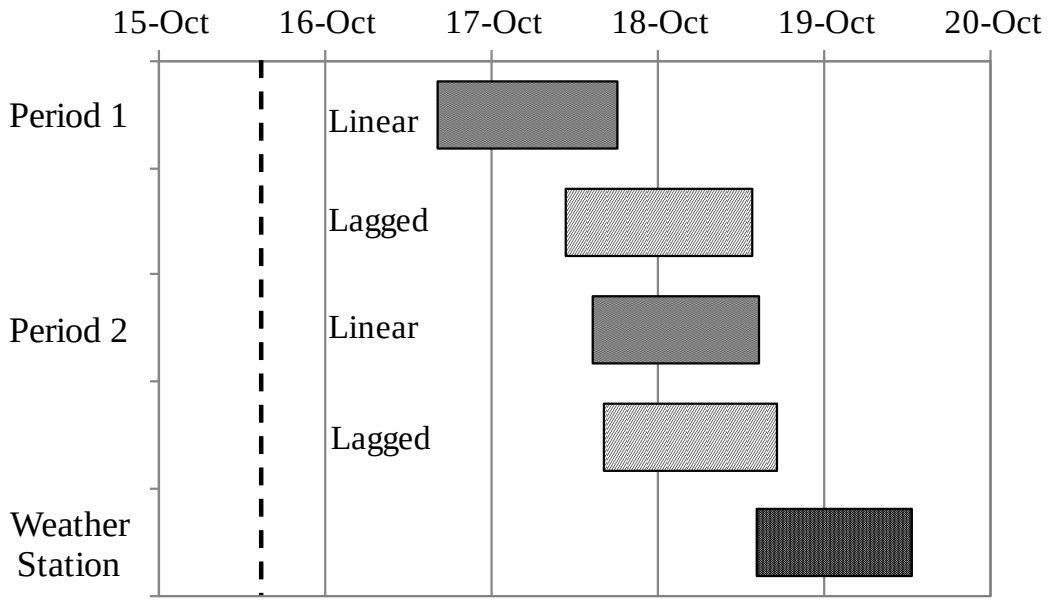


Figure 5. Minimum predicted range of colonization when estimated from linear and lagged regressions, by placing a data logger on-site at day 0 for 5 days (Period 1), at day 20 for 10 days (Period 2), or weather station data. Dashed vertical line represents when eggs were first noted on the carcass.

Discussion

Few studies have tested the accuracy of using regression analyses to predict body-recovery scene temperatures for use in the analysis of entomological evidence, and this study represents the first in North America to do so. Linear regression models allowed us to determine the minimum amount of hourly temperature data collection necessary to accurately represent on-site temperatures. If data collection began on the day the remains were found, recording hourly temperatures for a minimum of 5 days was sufficient. In contrast, Johnson et al. (2012) found that only 2 days of temperature collection were sufficient when collection began the days the remains were found. For our study, 3 days was insufficient, but this could be explained by the length of the body *in situ* period: we estimated temperatures for a 10 day *in* situ period and Johnson et al. (2012) estimated temperatures for 5 day in situ period. If data collection begins 20 to 60 days after discovery of the remains, recording hourly temperatures for a minimum of 10 days is sufficient, supporting the findings of Archer (2004). However, in contrast to Archer, weather station data were not a more accurate predictor than our model as elapsed time post-discovery increased. Perhaps this is because we simulated recording data for twice as long (up to 20 days vs. 10 days), or because we did not simulate recording data long enough after the hypothetical discovery of the remains (Archer (2004) simulated logger placement 78 days after discovery, whereas our maximum elapsed time was 60 days after discovery). However, we suspect that if too much time had elapsed, no duration of temperature recording would result in an accurate model.

Dabbs (2010) provided evidence that caution must be taken when choosing a weather station from which to obtain temperature data. In that study, a station located 9.9 km SE of

the study site recorded temperatures more similar to the test site than did a station located 5.7 km NE of the study site. This difference supports the use of regression analyses to predict on-site temperatures rather than using weather station data alone. Johnson et al. (2012) found no effect of distance on the accuracy of temperatures predicted by regression models, at distances up to 15 km away from the body-recovery sites. The differences between these two studies suggest that more work on how distance from a weather station might influence the accuracy of predicted temperatures is warranted.

Our data suggest that both linear and lagged regression models are useful for retrospective temperature estimation. The variation in mean absolute residuals across loggers and regression models shows the importance of placing a data logger as close to where the remains were located as possible. When testing the application of this study to entomological evidence collected in the field, we found that our models were more accurate than weather station data alone, providing support for this technique. Although all five predictions underestimated the actual time of oviposition, this can be attributed to the development data. The data set used (Byrd & Allen, 2001) is the most accurate in Raleigh, NC, but this underestimation also occurred in another study at the same location during the summer (Cammack, unpublished data). Further investigation is required to determine why lagged models were less accurate than linear models, particularly when based on data collected starting the day the remains were located. For both periods, the variance in the temperatures estimated from linear models was double that of the lagged models, suggesting that lagged models fail to account for the daily periodicity in temperature fluctuations. However, this effect is diminished as the amount of time between discovery and logger placement

increases, suggesting that lagged regression models might be useful only when a large amount of time has elapsed since discovery.

Future work should investigate the effect of season on the accuracy of this technique, as well as the use of different types of regression models for predicting on-site temperatures. We recommend that data logger placement and subsequent temperature recording should occur as soon after discovery of the remains as possible. However, our study indicates that useful data can be obtained up to 2 months after discovery of the remains, supporting the findings of other researchers (Archer, 2004; Johnson et al., 2012). The congruency between our study and those conducted in Australia indicate that this is a robust technique that will aid forensic entomologists in the analysis of entomological evidence.

References

Amendt, J. C.P. Campobasso, E. Gaudry, C. Reiter, H.N. LeBlanc, M.J.R. Hall. 2006. Best practice in forensic entomology – standards and guidelines. International Journal of Legal Medicine 121: 90-104.

Archer, M.S. 2004. The effect of time after body discovery on the accuracy of retrospective weather station ambient temperature corrections in forensic entomology. Journal of Forensic Sciences 49: 553-559.

Byrd, J.H. and J.C. Allen. 2001. The development of the black blow fly, *Phormia regina* (Meigen). Forensic Science International 120: 79-88.

Byrd, J.H. W.D. Lord, J.R. Wallace, and J.K. Tomberlin. 2010. Collection of entomological evidence during legal investigations. In: *Forensic Entomology: The Utility of Arthropods in Legal Investigations*, 2nd edition. J.H. Byrd and J.L. Castner, eds. CRC Press, Boca Raton, FL. Pp 127-175.

Catts, E.P. 1992. Problems in estimating the postmortem interval in death investigations. Journal of Agricultural Entomology 9: 245-255.

Catts, E.P. and M.L. Goff. 1992. Forensic entomology in criminal investigations. Annual review of Entomology 37: 253-272.

Dabbs, G.R. 2010. Caution! All data are not created equal: The hazards of using National Weather Service data for calculating accumulated degree days. Forensic Science International 202: e49-e52.

Higley, L.G. and N.H. Haskell. 2000. Insect Development and Forensic Entomology. In: *Forensic Entomology: The Utility of Arthropods in Legal Investigations*, 1st edition. J.H. Byrd and J.L. Castner, eds. CRC Press, Boca Raton, FL. Pp 287-302.

Johnson, A.P. J.F. Wallman, and M.S. Archer. 2012. Experimental and casework validation of ambient temperature corrections in forensic entomology. Journal of Forensic Sciences 57: 215-221.

National Research Council Committee on Identifying the Needs of the Forensic Sciences Community; Committee on Applied and Theoretical Statistics. 2009. Strengthening forensic science in the United States: A path forward. National Academies Press, Washington, D.C. 352 pp.

Saks, M.J. and J.J. Koehler. 2005. The coming paradigm shift in forensic identification science. Science 309: 892-895.

Scala, J.R. and J.R. Wallace. 2010. Forensic meterology: The application of weather and climate. In: *Forensic Entomology: The Utility of Arthropods in Legal Investigations*, 2nd edition. J.H. Byrd and J.L. Castner, eds. CRC Press, Boca Raton, FL. Pp 519-538.

Tarone, A.M. and D.R. Foran. 2006. Components of developmental plasticity in a Michigan population of *Lucilia sericata* (Diptera: Calliphoridae). Journal of Medical Entomology 43: 1023-1033.

Wells, J.D. and L. R. Lamotte. 2010. Estimating the Postmortem Interval. In: Forensic Entomology: The utility of arthropods in legal investigations, 2nd edition. J.H. Byrd and J.L. Castner, eds. CRC Press, Boca Raton, FL.

CHAPTER 4

An alternative diet for rearing blow flies (Diptera: Calliphoridae) in the laboratory

Jonathan A. Cammack[1*], Steve S. Denning[2], D. Wes Watson[3]
Department of Entomology, North Carolina State University, Campus Box 7626, Raleigh, North Carolina, 27695.

[1]jacammac@ncsu.edu,[*]corresponding author, [2]steve_denning@ncsu.edu, [3]wes_watson@ncsu.edu

Abstract

In this study, we investigated the utility of three alternative, oligidic diets (rehydrated dry fish, dog, and cat food) for rearing *Lucilia sericata* in the laboratory. Fish food was not a suitable diet, but dog food and cat food were. Pupae were significantly smaller when reared on these diets in comparison with control diets (beef liver and ground pork). However, diet had no effect on development rate or adult longevity, which suggests that dog and cat food are suitable alternatives for maintaining colonies of *L. sericata,* and other blow fly species. A diet of commercially-available cat food has been used exclusively to rear four additional blow fly species over a span of approximately 3 years. The ease of storage and preparation of these diets along with the reduction in odor when compared to traditional meat/tissue-based diets make these alternative diets advantageous to use for laboratory rearing of blow flies.

Introduction

Sarcosaprophagous in nature, blow fly larvae are typically reared on a raw animal-tissue-based diet, such as bovine or porcine organs (Anderson 2000; Clark et al., 2006) or ground muscle tissue (Byrd & Allen, 2001; Boatright & Tomberlin 2010). Although this method of rearing is simple and the diet easy to obtain, the associated putrefaction odors make it undesirable in a laboratory setting, and the tissue must be kept frozen for long-term

storage. Artificial diets have been developed and used to mass-rear blow flies, but these diets also contain raw animal tissue or blood (Taylor & Mangan, 1987; Daniels et al., 1991; Chaudhury & Skoda, 2007) and/or a large number of ingredients (e.g. Hodgson & Rock, 1971; see Singh, 1977 for a review) which require separate storage, measuring, and mixing for use. Although these diets work well for mass-rearing, their labor-intensive preparation makes them impractical for routine maintenance of small laboratory colonies (Mandeville, 1988). Several previous studies have used dry diets to avoid the pitfalls associated with raw, tissue-based diets. Dog biscuits soaked in water were used to rear larval blow flies (*Phormia regina* (Meigen)), but the effects on development and quality of the resulting adults was not reported (Frings, 1947). This diet was also suitable for rearing *Musca domestica* L. (Frings, 1948) and *Sarcophaga bullata* (Parker) (Frings & Frings, 1953). Chemical Specialties Manufacturers Association (CSMA) medium, a mixture of wheat bran, alfalfa meal, and brewers grain (Greenberg, 1954) was used to rear *M. domestica* and *Stomoxys calcitrans* (L.), but pupae of *M. domestica* produced on CSMA medium were smaller than those produced on other diets containing wheat bran, alfalfa meal, and other protein sources (Hogsette, 1992). *Lucilia sericata* (Meigen) has been reared on a diet containing a mixture of CSMA medium and Purina® Cat Chow (Mandeville, 1988). Although heavy mold growth on this diet affected larval development, when the mold growth was low, pupal weight and survival to the adult stage were not significantly different from flies reared on natural chicken liver and fish diets. A gelled diet containing egg powder, instant non-fat milk powder, and agar has been used to rear larvae of *L. sericata* and *Cochliomyia macellaria* (F.), but the effects of this diet on development and survival are unknown (Li. et al., 2013). The purpose of the current

study was to identify an oligidic diet for rearing blow fly larvae that was easy to obtain, inexpensive, required minimal preparation, and produced healthy flies. To identify this diet, we compared three commercially-available, dry, pet/animal feeds to two traditional animal tissue diets.

Methods and Materials

A three-year-old colony of *L. sericata* that originated in Morgantown, West Virginia was used for this study. The colony was maintained at approximately 22°C on a 16L:8D photoperiod, and adult flies were provided *ad libitum* with granulated sugar, powdered milk, and water. Larvae from the colony were reared on beef liver, the same medium which was used as an oviposition source.

To identify a suitable diet for laboratory rearing, larvae of *L. sericata* were allowed to develop on one of five diets: beef liver, ground pork, and rehydrated dry fish food (Cargill™ Fish Feed, Cargill, Inc., Minnetonka, MN, USA), dry dog food (Pedigree Puppy Chow®, Mars, Inc., McLean, VA, USA), or dry cat food (Friskies® Signature Blend). The fish food was used because this product was readily available at feed stores, and contains a high amount of protein and other ingredients that promote fish growth and muscle development. The dog and cat foods were chosen for their protein content and availability at local stores. Dry fish food was hydrated to 60% moisture by weight, and dry dog and cat food were hydrated to 70% moisture by weight, with distilled water. Adult flies were presented with

beef liver as an oviposition medium for approximately four hours. The liver was removed from the cage and incubated at 22°C for approximately 24hr. Ten first instars < 24 h old were placed on approximately 40 g of each diet treatment, in 120 ml specimen cups.

Moistened paper towels were placed on top of the diet to prevent desiccation, and cups of diet were inserted into a 355 ml plastic drinking cup so the diet cup was suspended approximately 25cm above 0.5 cm of dry soil in the bottom (Figure 1). The drinking cups were covered with a KimWipe™ laboratory tissue (Kimberly-Clark®) to allow for the passage of air and to prevent the contamination by other insects.

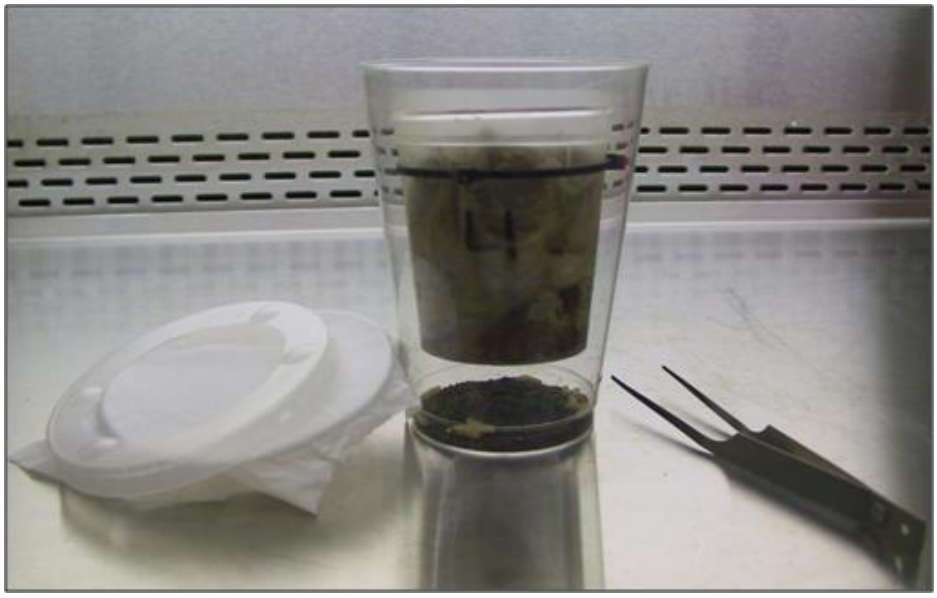


Figure 1: Rearing container, with pre-pupal larvae of *L. sericata* crawling on the soil surface.

Twelve cups (three of each diet) were used for each replicate. Four replicates of beef liver, ground pork, and dog food, two replicates of fish food, and two replicates of cat food were tested. Because the fly larvae did not consume the fish food, that treatment was only replicated twice and replaced with a cat food treatment for two replicates. Experiments were

conducted at 22°C and ~70%RH on a 16L:8D photoperiod. Pupal length and weight, as well as time to pupation, adult emergence, and death were recorded. Pupal length was recorded to the nearest mm with a digital caliper and weight to the nearest mg on an

analytical balance. After adult emergence, the flies were provided only water so that adult longevity could be used as an indicator of diet quality.

A separate study was conducted to test the utility of using dry dog or cat food as a substitute for powdered milk in the adult diet. Newly-emerged adults of *L. sericata* were provided water, and 6.5 g of a diet treatment containing equal parts of ground dog food plus sugar, ground cat food plus sugar, or powdered milk plus sugar. Flies were monitored three times daily for mortality until all flies were dead.

Data were analyzed with an ANOVA followed by Tukey-Kramer HSD test, in JMP Pro 9.0 (SAS Institute, Cary, NC).

Results

Diet had a significant effect on the size of pupae. Pupae were significantly longer ($F_{3,155} = 8.91$, $p < 0.0001$) (Figure 2) and heavier ($F_{3,155} = 14.14$, $p < 0.0001$) (Figure 3) when larvae developed on animal tissue diets (beef liver and ground pork) in comparison with alternative diets (dog food and cat food).

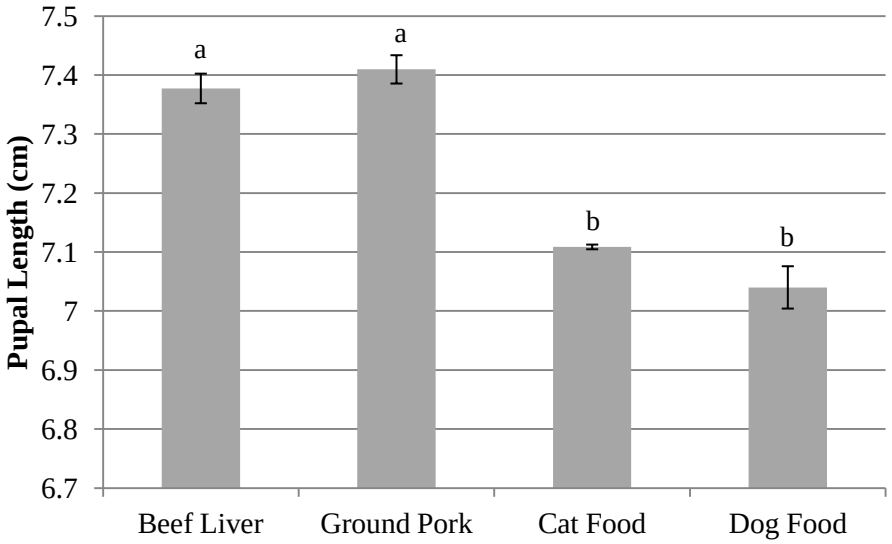


Figure 2. Mean length (mm) ± SE of pupae of *L. sericata* reared on four diets. Different letters indicate significant differences (α=0.05, Tukey-Kramer Honest Significant Difference Test).

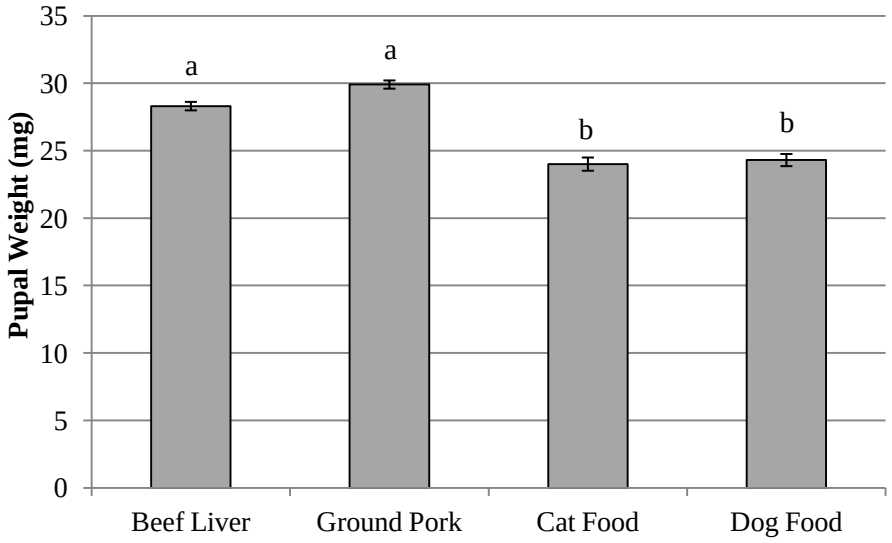


Figure 3. Mean weight (mg) ± SE of pupae of *L. sericata* reared on four diets. Different letters indicate significant differences (α=0.05, Tukey-Kramer Honest Significant Difference Test).

Adult flies lived approximately 18 hours longer when reared as larvae on alternative diets in comparison with animal tissue diets. However, diet had no effect on total development time ($F_{3,64} = 0.88$, $p = 0.45$), duration of the pupal stage ($F_{3,64} = 1.19$, $p = 0.32$), or adult survival under starving conditions ($F_{3,64} = 2.57$, $p = 0.06$). Percent emergence of adults was significantly affected by diet treatment ($F_{3,38} = 4.22$, $p = 0.01$) (Figure 4).

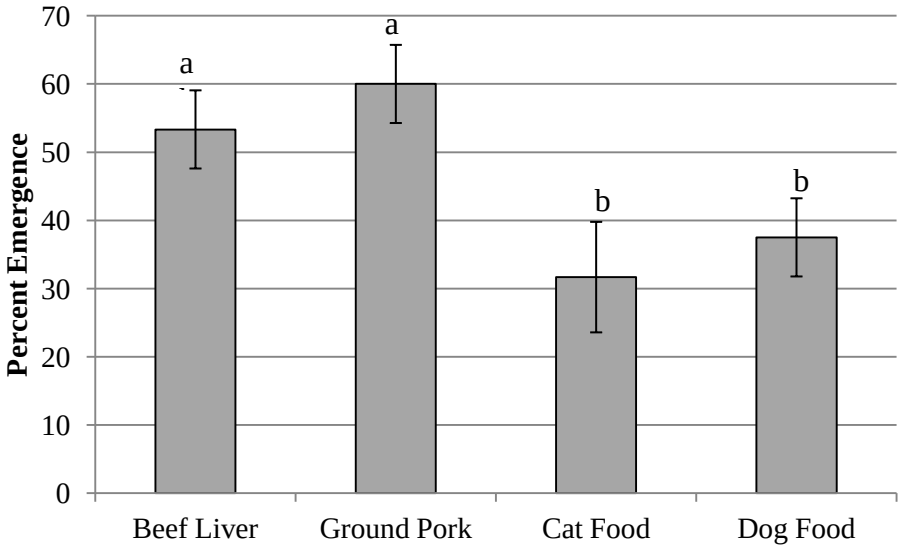


Figure 4. Percent emergence ± SE of adults of *L. sericata* reared on four diets. Different letters indicate significant differences (α=0.05, Tukey-Kramer Honest Significant Difference Test).

Adult flies lived significantly longer ($F_{2,119} = 124.17$, $p < 0.0001$) when provided with dry powdered milk and sugar, than when ground dry dog or cat food was used as a protein source to replace dry milk (Figure 5). Mean longevity of adult flies provided powdered milk and sugar was approximately 60 days, though some flies lived 70 days, and others a maximum age of 88 days.

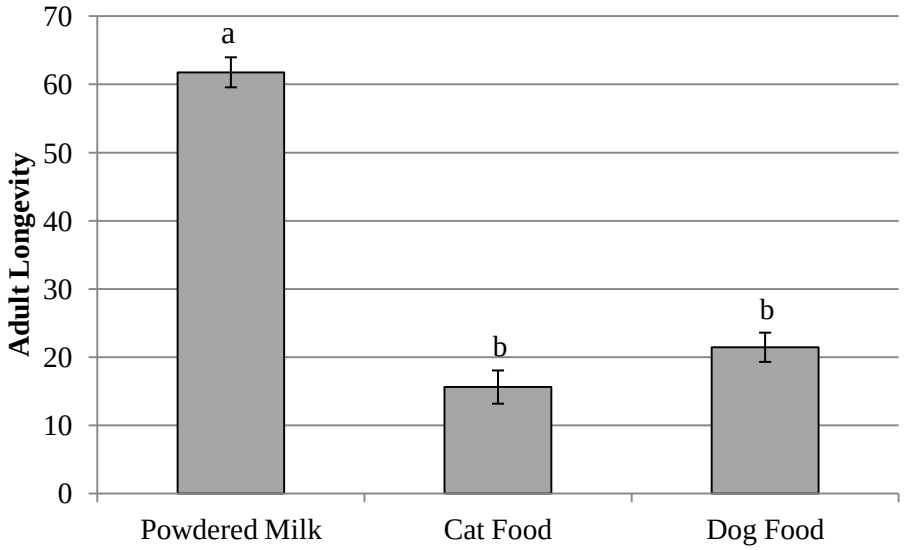


Figure 5. Life span of adults of *L. sericata* when reared on three different protein sources and granulated sugar. Different letters indicate significant differences (α=0.05, Tukey-Kramer Honest Significant Difference Test).

Discussion

This study indicates that dog food and cat food can be used to rear blow flies. Due to ease of preparation, cost, and availability, dry cat food was chosen as the food source for rearing blow flies in our colonies. Flies reared on the alternative diets were smaller than those reared on animal tissue diets, suggesting that dog food and cat food lack unknown dietary ingredients that optimize fly growth (Mandeville, 1988). Although diet significantly affected size, the lack of an effect on development and longevity suggests that these diets are suitable alternatives to traditional raw meat-based diets, especially if being used to maintain a colony. The results indicate that fly colonies may decline in number due to a negative effect on adult emergence; however, this was not noted when larvae were reared on a large scale on these diets. This study also indicates that when compared with dry dog or cat food, powdered milk

is a better protein source for maintaining long-lived adult flies in colony. However, the effects of this dietary protein source on reproductive capacity and gonotrophic development are unknown, which are greatly influenced by protein in the diet (Stoffolano, 1974).

In our study, as in Mandeville (1988), occasional fungal contamination of the medium occurred; however, no adverse effects on pupal length or weight, or development rate were noted. For rearing larvae to maintain our colonies, fungal contamination has been avoided by altering the cat food:water ratio by mixing equal volumes of cat food and water, and hydrating the medium overnight at 4°C. Insect dietitians frequently use the fungicide methylparaben to prevent fungal growth, but Voss (2000) indicated that methylparaben decreased development rate of fly larvae and increased pupal mortality of *Protophormia terraenovae* Robineau-Desvoidy. Therefore, caution should be used when adding methylparaben to the diet to prevent negative growth effects (Voss, 2000). Although the cat food used in this study was discontinued by Purina®, a new variety (Friskies® Surfin' & Turfin' Favorites) is a suitable alternative larval fly diet. *Protophormia terraenovae* was reared exclusively on this diet for approximately 1.5 yrs., and produced in excess of 100,000 flies. This cat food diet was also used to rear > 3,000 flies each of *Cochliomyia macellaria*, *Chrysomya megacephala* (F.), and *Phormia regina* Meigen. No negative effects on development, adult emergence, or adult size were noted in these four species, suggesting that these species might be better suited for being reared on cat food than the test species, *L. sericata*. For these authors, the odors associated with the dry dog and cat food diets were less offensive than those associated with beef liver or ground pork. Raw beef liver continues to be a preferred oviposition medium for our fly colonies, but *M. domestica* and *P. regina* have

been observed to oviposit on fermenting, moistened dog biscuit inoculated with yeast (Frings, 1948), and *L. sericata* on the gelled diet of Li et al. (2013), suggesting that blow flies could be maintained on an entirely meat-free diet. The low cost, availability, and minimal preparation of using dry cat food as a larval food source makes this alternative diet one that is suitable for rearing blow flies in the laboratory.

References

Anderson, G.S. 2000. Minimum and maximum developmental rates of some forensically important Calliphoridae (Diptera). Journal of Forensic Sciences 45: 824-832.

Boatright, S.A. and J.K. Tomberlin. 2010. Effects of temperature and tissue type on the development of *Cochliomyia macellaria* (Diptera: Calliphoridae). Journal of Medical Entomology 47: 917-923.

Byrd, J.H. and J.C. Allen. 2001. The development of the black blow fly, *Phormia regina* (Meigen). Forensic Science International 72: 79-88.

Chaudhury, M.F. and S.R. Skoda. 2007. A cellulose fiber-based diet for screwworm (Diptera: Calliphoridae) larvae. Journal of Economic Entomology 100: 241-245.

Daniels, S. K. Simkiss, and R.H. Smith. 1991. A simple larval diet for population studies on the blowfly *Lucilia sericata* (Diptera: Calliphoridae). Medical and Veterinary Entomology 5: 283-292.

Frings, H. 1947. A simple method for rearing blowflies without meat. Science 105: 482.

Frings, H. 1948. Rearing houseflies and blowflies on dog biscuit. Science 107: 629-630.

Frings, H. and M. Frings. 1953. Dog biscuit as a larval medium for Sarcophaga *bullata*. Journal of Economic Entomology 46: 183.

Greenberg, B. 1954. A method for the sterile culture of housefly larvae, *Musca domestica* L. The Canadian Entomologist.

Hogsette, J.A. 1992. New diets for production of house flies and stable flies (Diptera: Muscidae) in the laboratory. Journal of Economic Entomology 85: 2291-2294.

Li, F. S.P. Venski II, E. J. Belikoff, and M.J. Scott. 2013. Conservation and sex-specific splicing of the transformer Gene in the calliphorids Cochliomyia hominivorax, Cochliomyia macellaria, and Lucilia sericata. PLOS ONE 8: e56303.

Mandeville, J.D. 1988. rearing *Phaenicia sericata* (Diptera :Calliphoridae) on dry cat food with CSMA. Journal of Medical Entomology 25: 197-198.

Singh, P. 1977. Artificial diets for insects, mites, and spiders. IFI/Plenum Data Company. New York, New York. 594 pp.

Stoffolano, J.G. 1974. Influence of diapauses and diet on the development of the gonads and accessory reproductive glands of the black blowfly, Phormia regina (Meigen). Canadian Journal of Zoology 52: 981-988.

Taylor, D.B. and R.L. Mangan. 1987. Comparison of gelled and meat diets for rearing screwworm, *Cochliomyia hominivorax* (Diptera: Calliphoridae), larvae. Journal of Economic Entomology 80: 427-432.

Voss. M. 2000. Effects of methylparaben on the development and chemosensitivity of the blow fly Protophormia terraenovae Rob.-Desv. (Diptera: Calliphoridae). Canadian Journal of Zoology 78: 680-683.

CHAPTER 5

The negative consequences of insect domestication: Implications for forensic entomology.

Jonathan A. Cammack

Abstract

The application of medicolegal forensic entomology relies on the ability to accurately estimate the age of insect larvae collected from a corpse, and from this estimation, infer the minimum postmortem interval. This estimate is made under the assumption that data developed on laboratory-reared insects can be applied to insects collected in the field, and that temperature is the main variable responsible for growth. In this paper, we review the literature on insect colonization and laboratory rearing and show that other variables associated with insect rearing can be just as important as temperature in determining how fast an insect develops. The implications for forensic entomology are also discussed, and recommendations are made to advance research on the development of forensically-important insects.

Introduction

In recent years, the science behind many forensic science disciplines has come under question, with traditional identification sciences (e.g. fingerprint, bullet, and bite mark analysis) taking the brunt of this criticism. Much of this criticism has been to increase objectivity and scientific rigor, akin to that practiced in DNA analysis. However, despite major advances in theory (e.g. the *Daubert* standard, on the admissibility of scientific evidence and expert testimony), relatively little research has addressed the accuracy of

traditional forensic sciences (Saks and Koehler, 2005), including entomology. In 2009, the National Research Council published a report (NRC, 2009) indicating that many forensic sciences needed improvement in order to meet the requirements of the *Daubert* standard. The NRC report emphasized that disciplines within the forensic sciences could be improved through basic research and scientific practices formed from testable hypotheses, valid statistical analysis, and peer review.

The ecological process of decomposition is comprised of a complex series of cascading events involving abiotic and biotic factors that contribute to the reduction of biomass to its basal elements. Insects often play a role in the decomposition process. Tomberlin et al. (2011) created a diagram of the decomposition process with respect to insect activity in order to help define decomposition events (Fig. 1). Of particular interest are two specific intervals: the precolonization and postcolonization intervals (pre-CI and post-CI, respectively), which make up the postmortem interval (PMI). The pre-CI is difficult to estimate because of many unknown response variables; for example, unknown response times by female blow flies; which can be affected by the distance between a female fly and a corpse, as well as the environment or concealment of the remains (Erzinclioglu, 1985; Goff 1991, 1992; Pujol-Luz et al., 2008). In contrast, the post-CI has been the focus of many studies, especially on larval development rates (Catts and Goff, 1992), which is used to estimate the age of insects collected from a corpse and provide an estimation of the minimum post mortem interval (mPMI).

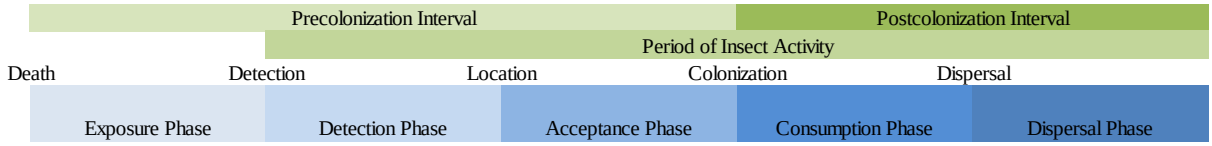


Figure 1. Stages of the decomposition process, with respect to insect activity. Modified from Tomberlin et al. (2011).

Development data sets are available for a subset of forensically-important species (Table 1) and have been developed from different populations worldwide. A comparison of data sets for the same species reveals a large amount of variation even when the parent populations were from similar climates (e.g. *Cochliomyia macellaria* (Fabricius) from Texas (Boatright & Tomberlin, 2010) and Florida (Byrd & Butler, 1996), suggesting that some underlying mechanism is responsible for the differences. Perhaps the most well-known example of how different data sets affect forensic entomology and the judicial process was the trial of David Alan Westerfield in the murder of Danielle van Dam (VanLaerhoven, 2008).

Table 1. Forensically-important calliphorid species for which development data have been generated from North American populations.

Species	Reference	Colony Origin
CALLIPHORINAE		
Calliphora latifrons	Anderson (2000)	British Columbia, Canada
	Kamal (1958)	Washington
Calliphora terraenovae	Kamal (1958)	Washington
Calliphora vicina	Anderson (2000)	British Columbia, Canada
	Greenberg (1991)	Illinois
	Kamal (1958)	Washington
Calliphora vomitoria	Kamal (1958)	Washington
Cynomya cadaverina	Kamal (1958)	Washington
CHRYSOMYINAE		
Cochliomyia macellaria	Boatright and Tomberlin (2010)	Texas
	Byrd and Butler (1996)	Florida
Chrysomya rufifacies	Byrd and Butler (1997)	Florida
Phormia regina	Anderson (2000)	British Columbia, Canada
	Byrd and Allen (2001)	Florida
	Greenberg (1991)	Illinois
	Kamal (1958)	Washington
	Nabity et al. (2006)	Nebraska
Protophormia terraenovae	Kamal (1958)	Washington
LUCILINAE		
Lucilia cuprina	Ash and Greenberg (1975)	Illinois
Lucilia illustris	Anderson (2000)	British Columbia, Canada
Lucilia sericata	Anderson (2000)	British Columbia, Canada
	Ash and Greenberg (1975)	Illinois
	Greenberg (1991)	Illinois
	Kamal (1958)	Washington

In People v. Westerfield, four entomologists provided estimates of the mPMI; Robert Hall, Neal Haskell, David Faulkner, Lee Goff and. Courtroom transcripts (Union-Tribune, 2002) suggest that each used different data and calculations, resulting in mPMI estimates that overlapped by 3-7 days. The estimate provided by Dr. Robert Hall was based on three data sets: Anderson (2000); Greenberg (1991) and Kamal (1958), for three species *Phormia regina*, *Lucilia sericata*, and *Calliphora* sp. Applying local temperature data from two weather stations (Singing Hills Country Club and Brown Field) to the published development data for each species produced overlapping estimates of colonization. Hall's estimate was the most conservative (i.e.11 days), and his use of three data sets was appropriate, given that he had no reason for choosing one data set over another. Dr. Neal Haskell used Kamal (1958) for *P. regina* (N.H. Haskell, personal communication) and temperature data from Brown Field to develop his estimate of colonization. Haskell's estimate of 9 days overlaps that of Hall's. Presumably David Faulkner, a forensic entomologist from San Diego, where the murder occurred, would have been familiar with localized developmental rates for these species. Faulkner's short estimate of 3 days falls within the timelines provided by Drs. Hall and Haskell, and information from the courtroom transcripts suggests he used the data of Greenberg (1991), and temperature data from Lindberg Field and El Cajon. Dr. Lee Goff's estimate of colonization encompassed 5 days but was 3 days prior to Hall's earliest estimate. Courtroom transcripts suggest he used the data of Anderson (2000) and temperature data from Singing Hills Country Club. Hall's subsequent analysis of Goff's report indicated a calculation error resulting in the discrepancy. Despite the use of different data sets and weather stations, and calculation errors, the resulting estimates were remarkably consistent.

However the documented differences of opinion and analysis point to a need for standardization and validation of methods employed in this trial. A decade later, these needs are still unmet.

Two studies have proposed that the mechanisms responsible for the differences between data sets might be the interaction between genetics (Picard and Wells, 2010) and environmental (rearing) conditions (Tarone and Foran, 2006). In this paper, we present a review of insect colonization (domestication) literature as it relates to forensic entomology, in an attempt to elucidate the many factors that alter the development of blow flies. Implications for the field of forensic entomology also are discussed, as well as recommendations for advancement of the field.

Development of Insects

Growth and development in insects is a complex biological process involving 3 to 4 predetermined life stages (e.g. egg, larva, pupa, and adult stages in holometabolous insects). Insects are ectothermic, therefore the rate at which they develop is dependent on the rate at which the enzymatic processes responsible for their growth and development are activated (Sharpe and DeMichele, 1977). At lower temperatures, the rate of development decreases, and the opposite is true at higher temperatures. However, insect growth and development is not dependent exclusively on temperature, because even under optimal conditions development can be highly variable.

One of the most widely recognized temperature-independent factors associated with insect development is the sex of the individual insect. The most common way sex affects development in insects is a biological phenomenon known as protandry, where males emerge

from the pupa sooner than do females (Buck, 2001). The ultimate cause of protandry is poorly understood across insects (Matsuura, 2006), but the mechanism responsible could be sexual selection (males increasing their likelihood of encountering females (Wiklund and Solbreck, 1982)), a female reproductive strategy (reduction in duration of the premating period (Fagerström and Wiklund, 1982)), or sexual size dimorphism (females are typically larger as a result of prolonged development time (Thornhill and Alcock, 1983; Matsuura, 2006)). When larvae of *Lucilia sericata* (Meigen) in the dispersing phase were placed on soil and allowed to complete development, males emerged significantly sooner (mean of 7.1 hours) than females (Cammack, 2009). However, information about sex-specific emergence is rarely presented in published development studies, as the development time is presented as the amount of time for a cohort to complete development (e.g. Byrd and Allen, 2001; Boatright and Tomberlin, 2010). Presentation of development data based on sex is extremely important for species such as *Chrysomya rufifacies* (Macquart), where females lay single-sex egg batches (Baumgartner, 1993).

Circadian rhythms also can influence development, through a phenomenon known as gating, where biological processes/events are influenced by environmental factors such as photoperiod (Beck, 1980). When pupae of *Drosophila pseudoobscura* Frolova and Astaurov that completed larval development under constant light were transferred to complete

darkness to synchronize their circadian rhythms, development of morphological characters was timed based on time of pupation, but eclosion of the adult was not. Adults emerged in three cohorts, each separated by approximately 24 hours (Beck, 1980). Similar results were obtained when the insects were maintained under a 18:6 (L:D) photoperiod;

morphological development was timed based on pupation, but adults emerged shortly after the initiation of photophase in two cohorts separated by approximately 24 hours (Beck, 1980). Some development studies on forensically-important species have been conducted under continuous light and constant temperature (e.g. Greenberg 1991; Byrd and Butler, 1996, 1997, 1998; Byrd and Allen, 2001) to prevent emergence gating. Other studies use different photoperiods (Byrd and Butler 1996, 1997, 1998 (all temperatures tested except 25°C); Byrd and Allen, 2001 (all temperatures tested except 25°C); Boatright and Tomberlin, 2010 (14L:10D)), or do not present photoperiod at all (Anderson, 2000; Grassberger and Reiter 2001, 2002). None report on whether or not emergence gating occurred. Eclosion from the egg, larval ecdysis, and pupation are also gated in some species (Beck, 1980), suggesting this might also occur in forensically-important species.

The role of critical weight in insect development is an important consideration for forensic entomology. Critical weight is the minimum mass beyond which no further growth is required for pupation to occur. At critical weight, juvenile hormone esterase is secreted, breaking down juvenile hormone, and the presence of elevated prothoracicotropic hormone (PTTH) and ecdysteroids at this time induces metamorphosis (pupation) (Davidowitz et al., 2003). For example, four species of blowflies (*Chrysomya albiceps* (Wiedemann), *Chrysomya megacephala* (Fabricius), *Lucilia eximia* (Wiedemann), and *Lucilia cuprina* Wiedemann) successfully pupated and eclosed as adults only when a minimum weight of 30 to 35 mg was reached (on both an artificial diet and ground beef) (Ribeiro and Von Zuben ,2010). Similarly, *Chrysomya megacephala* pupated and successfully eclosed only if a minimum weight of 30.5 mg was reached (Von Zuben 1998). Seven calliphorid species in

Australia exhibited similar minimum weights requirements for successful eclosion, ranging from 13.4 to 100.9 mg, with five of the seven species weighing between approximately 33 and 40 mg (Levot et al., 1979). However, these recorded weights are not true critical weights, as when the larvae were weighed, they had already entered the dispersing phase, and thus were exhibiting behaviors of blow flies ready to pupate. The critical weight in *Cochliomyia hominivorax* (Coquerel) is probably between 30 and 40 mg, and larvae weighing near 40 mg produced adult females with the highest percentage of viable ova (Hightower et al., 1972). Critical weight is a complex phenomenon affected by environmental stimuli, such as temperature. For example, larvae of *Cochliomyia macellaria* reared at 15.6°C reach a smaller length (and thus lower weight) than do those reared at 32.2°C (Byrd and Butler, 1996). In contrast, larvae of *Sarcophaga haemorrhoidalis* Fallén reach approximately the same maximum length regardless of rearing temperature (Byrd and Butler, 1998). An alternate explanation is that critical weight is independent of temperature, and the maximum size reached is temperature-dependent in the sense that the enzymatic processes responsible for food digestion and growth are slowed at lower temperatures, and thus larvae are not able to convert as much food to biomass before PTTH and ecdysteroids initiate pupation behaviors. Critical weight is also affected by food availability and quality, dehydration, gates, sex, and other factors (R.M. Roe, personal communication).

Founder Effect and Laboratory Rearing

The amount of genetic diversity in any population is directly related to the size of that population. In wild populations, population size (and its inherent genetic diversity) can increase as individuals immigrate into the population and outbreeding occurs (also known as

gene flow) (Wilson and Bossert, 1971). When a laboratory colony is established, the number of individuals collected is much smaller than the natural, wild population. This establishment of a population with a smaller number of individuals that carry a fraction of the genetic variability of the parent population is known as founder effect (Wilson and Bossert, 1971). Bringing these insects into the laboratory results in a closed population and subsequent changes in genetic variation will be made from the decreased amount of genetic material in the new population (Bartlett, 1985). The maintenance of a closed population for even a short period of time can result in a loss of genetic diversity. In a laboratory population of *Lucilia sericata*, the number of alleles present at nine loci declined in six generations, from 39 to 24 (a reduction of 38%). During the six generations, alleles were lost at eight of the nine microsatellite loci tested (Florin and Gyllenstrand, 2002).

Perhaps the most well-known example of a change in allelic diversity in blow fly colonies is that of α-glycerol dehygrogenase (α-GDH) in *Cochliomyia hominivorax*. Two allelic forms of this enzyme are present in natural populations, and are responsible for regulating energy flow required for flight (Bush et al., 1976). In mass-culture, flies were kept in large cages with newspaper suspended from the ceiling of the cage, which increased surface area for resting and prevented flight. Placement of food and oviposition sources on the floor of the cage selected for flies that walked or flew short distances. This resulted in fixed or nearly-fixed frequencies (0.89-1.00) of the α-GDH_1 allele in three of the strains used for the screwworm eradication program. Another strain, which was a hybrid of two laboratory strains had low frequencies (0.31) of α-GDH_1 when started. Within 15 generations of mass production, the frequency had risen to 0.74 (Bush et al., 1976). This change occurred

even though colonies were maintained in high numbers (~70,000/ cage), suggesting that colony size is not the only factor governing genetic diversity.

The geographic location from which founding individuals are collected can also affect the amount of genetic diversity within a colony. According to Bartlett (1985), if a species is highly mobile and widely distributed (e.g. blow flies), collection from a single or few geographic locations should be representative of the entire population. However, collection of individuals from a single location for establishing a colony (Wells and Kurahashi, 1994; Tachibana and Numata, 2004; Nabity et al., 2009) might not be representative of the genetic diversity within a wild population. Adults of *Phormia regina* (Meigen) collected at a decayed meat bait within 30 minutes of placement in 15 locations across the U.S. displayed a high degree of relatedness within each sample (mean relatedness coefficient ranged from 0.05-0.79) (Picard and Wells, 2009). A similar trend was found in adults of *Lucilia sericata* collected in 10 locations across the U.S. and Canada; the mean relatedness coefficient within samples ranged from 0.08-0.72 (Picard and Wells, 2010). In two samples of *P. regina* from West Virginia, the mean relatedness within a sample of collected flies was greater than that of full siblings generated in the laboratory. The authors hypothesized this was a result of the reproductive behavior of blow flies. A female blow fly can deposit hundreds of eggs (Mackerras, 1933; Yin and Stoffolano, 1998; Tarone and Foran, 2006), resulting in a large number of siblings that complete development and emerge together. If these individuals are attracted to the same bait and collected to start a colony from which development data will be generated, the resulting colony will be inbred and genetically homogenous (Picard and Wells, 2009). In contrast, collection of individuals at

locations separated by relatively small distances (e.g. ~125 miles) can result in genetic variation (Picard and Wells, 2009).

Laboratory Rearing Parameters Affecting Insect Development

The application of medico-legal forensic entomology operates under a major assumption that published development data sets (particularly for blow fly species) that were generated from laboratory colonies can be applied to wild populations. However, colonizing a species in the laboratory puts selection pressures on the laboratory population that may result in a colony that is very different from wild populations, particularly in terms of rate of development, which is of interest to forensic entomology. These differences also may become exacerbated as the age of the colony increases. This "colony effect" is often anecdotally stated as the reason why one of the oldest data sets (Kamal, 1958) underestimates larval age, and subsequently the minimum post-mortem interval, mPMI. This phenomenon of increased rate of development in species in colony has been noted in numerous arthropod species, from mites to higher Diptera.

Evidence from non-forensically important species

In the European House Dust Mite, *Dermatophagoides pteronyssinus* (Trouessart) (Acari: Pyroglyphidae), development of eggs from a seventeen-year old laboratory colony and a wild population (in colony for one month) was compared at 30°C and 80% relative humidity (Colloff, 1987). Eggs from the laboratory population developed significantly faster ($p < 0.01$) than the wild population (mean of 3.9 and 5.13 days, respectively). Development from egg-adult in the Indian meal moth, *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae) was significantly different between a 20+ year-old laboratory colony and a wild-

type population that was collected approximately 160 km from the collection site of the laboratory colony (Johnson et al., 1995). On 5 of 6 diets tested, the mean development requirement in accumulated degree days was faster for the laboratory colony than the wild-type, and this difference was significant ($p = 0.0001$) for 4 of the 6 diets (Table 2).

Table 2. Difference in the rate of development from egg to adult between laboratory and wild-type populations of *Plodia interpunctella*.

	Mean Accumulated Degree Days (±SE)		Difference[1] in Development Rate
Diet	Laboratory Colony	Wild-type	(ADD)
Wheat Bran	345 ± 2.32	844 ± 2.25	-499*
Pistachios	574 ± 2.05	1036 ± 1.39	-462*
Walnuts	613 ± 2.91	1092 ± 1.72	-479*
Almonds	536 ± 6.53	1082 ± 1.06	-546*
Raisins	1241 ± 112.43	1382± 32.35	-141
Prunes	1616 ± 224.55	1383 ± 13.66	233

[1] Expressed as ADD of the laboratory colony minus ADD of the wild-type colong. * = significant difference between the two populations ($p = 0.0001$). Modified from Johnson et al. (1995).

Development of the chironomid midge, *Chironomus tepperi* Skuse, is affected by both temperature and colonization (Stevens, 1993). When reared at 25°C, development from egg-adult required 13 days for both wild-collected eggs and eggs from a sixth-generation colony. In contrast, at 17.5°C, wild-collected eggs required 24 days to develop to the adult stage, and eggs from an F_6-colony required 19 days to develop to the adult; a difference of five days. Interestingly, all of this five-day difference was seen as an increase in the length of the pupal stage.

The most numerous and profound examples of the effects of colonization is with the fruit fly family, Tephritidae. Species in this family have been colonized and mass-reared to

develop control methods for their wild counterparts, which are pests of numerous crops (Tsiropolous, 1992). In some cases, these colonies have been maintained for many years and generations (over 300 in some cases). Time of development from egg to pupa in the Oriental fruit fly, *Dacus dorsalis* Hendel, was different between a lab colony that was approximately 300 generations old and progeny of wild-caught individuals (Foote and Carey, 1987). On four of seven diets tested, larvae from the laboratory colony developed faster (mean of 1.625 days) than did larvae of wild-caught adults (Table 3).

Table 3. Difference[1] in the duration of larval development (days) between laboratory (~F_{300}) and wild (F_1) populations of *Dacus dorsalis*. [1] Expressed as larval development time of F_{300} minus larval development time of F_1. Modified from Foote and Carey (1987).

	Mean Development Time (d ± SD)		
Diet	Laboratory Colony	Wild	Difference in Development Time (d)
Artificial Wheat Diet	9.0 ± 0	10.5 ± 1.47	-1.5
Avocado	9.0 ± 0.71	8.7 ± 0.44	0.3
Banana	18.0 ± 1.08	11.8 ± 1.31	6.2
Fig	10.7 ± 1.06	12.9 ± 1.42	-2.2
Mango	12.2 ± 0.64	13.4 ± 2.39	-1.2
Papaya	9.5 ± 1.28	11.1 ± 1.32	-1.6
Peach	12.1 ± 0.93	10.1 ± 0.93	2.0

A similar trend was found in the Mediterranean fruit fly, *Ceratitis capitata* Wiedemann (Bravo & Zucoloto, 2003). Laboratory colony flies (F_{160}) developed significantly faster ($p < 0.05$) on papaya and an artificial diet (by 1.2 and 3.7 days, respectively) than progeny (F_1) from wild-caught adults.

Evidence from forensically important species

No direct comparison of development rates between laboratory colonies and wild populations could be found in the calliphorid literature. However, one study (Nabity et al., 2006) on the development of *Phormia regina* reported differences between replicates, where F_8 and F_3 individuals were used for the study. Flies for both colonies were collected from the same location, but 3 years apart. At two of the mean temperatures tested (25.5°C and 31°C, temperatures at which this species would be active during most of the year throughout its distribution, F_8 flies developed faster from both egg to pupa and egg to adult, than the F_3 flies (Table 4). Although this difference was not statistically significant, a difference in development time (and subsequent estimate of the mPMI if using total development time) of 1.3 to 1.5 days could have major implications in a forensic context.

Table 4. Difference in development time (days) from egg to pupa and egg to adult between F_8 and F_3 colonies of *Phormia regina*. *Some temperatures are presented as a mean because the temperatures at which the incubators were set differed between years one and two (31=32 in year one, 30 in year two; 25.5=26 in year one, 25 in year two). [1] Expressed as development time of F_8 minus F_3. Modified from Nabity et al. (2006).

	Difference[1] in Development Time (d)	
Mean Temperature (°C)*	Egg-Pupa	Egg-Adult
31	-0.9	-1.5
25.5	-2.0	-1.3
20	1.9	1.4
14.5	2.1	6.2

Colony age and inadvertent selection might offer an explanation for the differences seen between published development data on *Phormia regina* (Meigen) (Fig 2). Data from

Kamal (1958) are based on F_{23} individuals and Byrd and Allen's (2001) on F_3 individuals. The number of generations of laboratory rearing for Greenberg (1991) is unknown. Although the rearing temperatures differ between these studies, the correlation between development time and rearing temperature is opposite to what would be expected. Kamal (1958), where flies were reared at the lowest temperature, would be expected to develop the slowest, and Byrd & Allen's (2001) (reared at the highest rearing temperature) would be expected to develop the fastest.

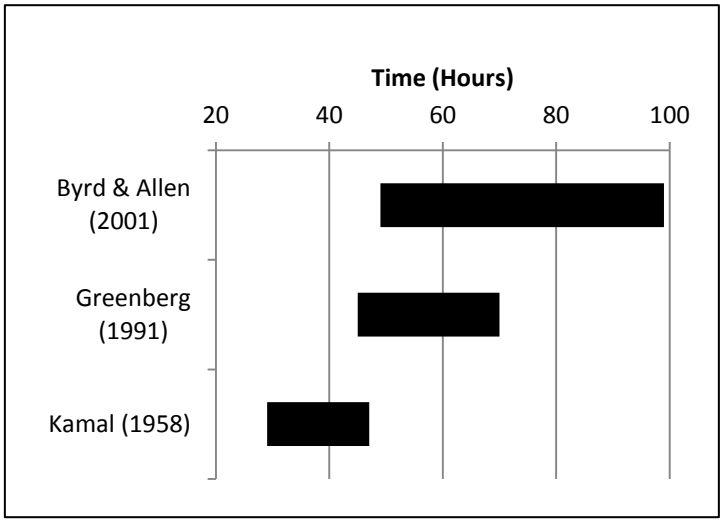


Figure 2. Minimum time (hours) for *Phormia regina* to enter and complete the 3rd stadium as calculated from three data sets. Rearing temperature (°C) for Byrd & Allen (2001) was 30°C, Greenberg (1991) was 29°C, and Kamal (1958) was 26.7°C.

Geographic differences between strains might offer some explanation, but cannot explain all the variation. Kamal's and Greenberg's colonies were started from flies caught in Colorado and Illinois, respectively; while Bryd and Allen's colony was established from flies caught in Florida. Colorado and Illinois are a higher latitude than Florida, and research on

Chrysomya megacephala suggests that flies from higher latitudes take longer to develop than do flies from lower latitudes (Hu et al., 2010).

Potential Sources of Variability in Development

Many factors associated with laboratory rearing contribute to the development of insects (Fig 3), ranging from genetic homogeneity that resulted from bottlenecking as discussed previously, to the methods under which the colony was maintained. This section will focus on how rearing techniques affect the development of insects, under the hypothesis that acclimation to laboratory rearing conditions is responsible for differences seen between laboratory and wild populations.

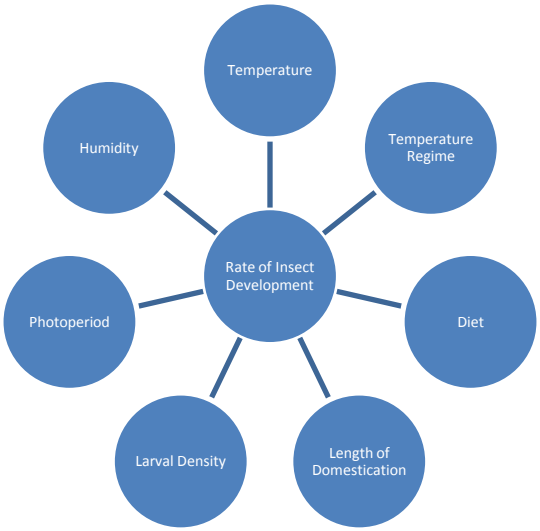


Figure 3. Some human-mediated factors associated with laboratory rearing that affect the rate of insect development.

The diet on which insects are reared has a significant impact on their rate of development, and this is seen in species of both agricultural and forensic importance. Indianmeal moths develop at significantly different ($p = 0.0001$) rates on artificial and natural diets, regardless of colony age; a twenty year old colony or the wild (Table 5) (Johnson et al., 1995).

Table 5. Mean accumulated degree days (ADD) from egg to adult for two isolates of *Plodia interpunctella*, reared on different diets. Different letters within a column indicate significant differences between diet treatments. Modified from Johnson et al. (1995).

	Mean ADD ± SE	
Diet	Laboratory	Wild-type
Wheat Bran	345 ± 2.32a	844 ± 2.25a
Pistachios	574 ± 2.05c	1036 ± 1.39b
Walnuts	613 ± 2.91d	1092 ± 1.72d
Almonds	536 ± 6.53b	1082 ± 1.06c
Raisins	1241 ± 112.43a	1382 ± 32.35a
Prunes	1616 ± 224.55a	1383 ±13.66a

More importantly, for forensic entomology, this phenomenon has also been noted in blow flies. *Calliphora vomitoria* L. develops at different rates when reared on different diets, i.e., porcine liver, muscle, and brain tissue (Ireland & Turner, 2006). Across densities ranging from 1-100 larvae/ 10g wet food, *C. vomitoria* completed development in 21.31, 21.75 and 23.17 days, on each respective tissue. *Lucilia sericata* developed at significantly different rates when reared on porcine and bovine liver, heart, and lung tissue (Clark et al., 2006). On average, larvae that fed on heart and lung reached the dispersing stage 31.0 and 29.2 hours

sooner than larvae feeding on liver; however, tissue type had no effect on the amount of time from dispersal to emergence. Differential rates of development also occur when blow flies are reared on tissue from different species. *Lucilia sericata* reached the dispersing stage significantly faster when reared on bovine tissue than on porcine tissue (range of approximately 2-20 hours, depending on tissue type) (Clark et al., 2006).

The temperature regime (constant vs. fluctuating) under which blow flies develop also affects the rate of development. For development studies, blow flies are typically reared at a constant temperature (Boatright & Tomberlin, 2010; Byrd & Butler, 1996; Nabity et al. 2006) unlike the cyclic temperatures in nature. *Phormia regina* develops slower when reared on ground lean pork, under fluctuating temperatures, in comparison to constant temperatures (Table 6) (Byrd & Allen, 2001). Under fluctuating temperatures (a 10°C around the same temperatures as the constant rearing temperatures), all development stages were longer, with most of the difference occurring in the third stadium (both feeding and dispersing stages) and pupal stages.

Table 6. Time (hours) for *Phormia regina* to reach the prepupal and adult stages at three different mean temperatures, under two temperature regimes. * Oscillation of 5 degrees above and below the mean temperature. Modified from Byrd and Allen (2001).

	Time (hours) to reach stage:			
	Prepupal (dispersing)		Adult	
Mean Temperature	Constant	Fluctuating*	Constant	Fluctuating*
40	104.8	166.1	378.8	647.1
30	124.4	177.9	480.4	667.9
20	188.2	321.5	813.4	1064.5

As previously discussed, photoperiod can have profound effects on development, especially in terms of emergence gating. Additionally, photoperiod also influences development rate in blow flies. When reared at 20°C or 25°C under a photoperiod of 12L:12D or 24L:0D, *Phormia regina* developed significantly faster ($p = 0.0155$ and 0.0146 for each respective temperature) under cyclic than continuous light (Nabity et al., 2007). In a meta-analysis of two studies on *P. regina*, development from egg to pupation and pupation duration occurred significantly faster ($p = 0.0088$ and $p = 0.047$, respectively) under cyclic than continuous light (Nabity et al., 2007). Though poorly documented, light quality and intensity also influence development rate (A.C. Cohen, personal communication).

Crowding during the larval stage also has an effect on the rate of development. In general, as larval density increases, the rate of development decreases. Depending on tissue type, *Calliphora vomitoria* develops faster by approximately two to three days when reared at a density of 10 or 100 larvae per 10 g of food (Ireland and Turner, 2006). *Calliphora vicina* Robineau-Desvoidy is affected in a similar manner when reared at 50 or 150 larvae per 50 g of food (Saunders and Bee, 1995). Crowded larvae developed significantly faster from egg to dispersal, pupation, and eclosion ($p < 0.01$, $p < 0.001$, $p < 0.001$, respectively). A similar trend was found when larvae of *Protophormia terraenovae* (Robineau-Desvoidy) and *Sarcophaga bullata* Parker were reared at different densities (Rivers et al., 2010). For both species, the larval stadium was significantly shorter ($p < 0.05$) as density increased.

Presumably the factors discussed here are not the only mechanisms responsible for the increased rate of development in colonies, as insect rearing is a complex system involving the manipulation of many variables. Human selection also might provide an

explanation. Removal of the oviposition media too soon (and thus excluding late or slow ovipositors), collecting only early pupae, or not allowing all adults to eclose, could, over time, result in a colony that produces adults that oviposit sooner and immatures that develop faster.

Implications for Forensic Entomology

The previous sections have illustrated that development in domesticated insect populations (i.e. laboratory colonies) is a complex biological process, influenced by both genetics and laboratory rearing practices. Combinations of these variables associated with insect rearing (temperature, temperature regime, photoperiod, diet, etc.) are extremely diverse in the literature. However, due to the logistics of rearing capabilities in *every* facility, no study or rearing operation can encompass all of the parameters that can influence insect development. Therefore, the assumption that insect age, and thus colonization of a body, can be accurately predicted based on temperature alone, when the other variables associated with development may be just as important, is a shortcoming. Given that many of these variables change the rate of insect development, estimates of larvae age are, at best, under-predictions. Also, assuming the validity of a data set developed from a geographically distinct population from one in question for case work is flawed.

VanLaerhoven (2008) addressed the flaws in these assumptions by conducting a validation study of published development data sets for *Phormia regina*. In this study, using the data of Anderson (2000), with a minimum threshold of 0°C, provided the most accurate estimate as to when colonization of pig carcasses occurred. This data set provided estimates of colonization the encompassed the actual date of death for all three case studies. Analysis

based on the data of Byrd and Allen (2001) accurately estimated colonization on two of the three carcasses. From this study, the author now has a scientific basis for using a particular development data set and minimum development threshold for casework. Choosing the incorrect data set and development threshold combination would result in an overprediction of the onset of colonization by up to nine days, or an underprediction by up to six days.

Future Steps

Colony establishment affects the bionomics of insect species, specifically their rate of development. Forensic entomologists must be aware of this, as a vast majority of the application of forensic entomology relies on this one parameter. Genetic factors (such as bottle-necking and the ability to survive and reproduce in colony) are likely the causes for the differences seen between laboratory and wild populations of insect species. Unfortunately, no studies have addressed these mechanisms which likely underlie the proximate mechanisms discussed in this paper.

In light of the information presented in this paper, the following recommendations are proposed to advance the use and practice of medicolegal forensic entomology:

1. Research colonies should be established from individuals collected in multiple locations, at different time periods. This will prevent the collection of only highly-related individuals, and result in a colony with increased genetic diversity.
2. Development data sets should be generated from individuals as close to wild as possible, with F_1 being ideal.
3. Research, develop, and publish development data for common species for which no data currently exist (e.g. *Lucilia coeruleiviridis*).

4. Blow fly larvae should be reared on a substrate that closely approximate their natural food source (e.g. a mixture of organ and muscle tissue, or organ and muscle provided at different times to represent patterns of consumption of remains.
5. The temperature regime under which development data are generated should fluctuate, to mimic that in the natural environment.
6. The photoperiod under which development data are generated should consist of both a photophase and scotophase. Although constant light is used to avoid emergence gating, constant light is never encountered in nature. Studies should be conducted to determine if the time range of adult eclosion is different between constant light and a L:D regime.
7. Forensic entomologists should validate data sets in the geographic area in which they practice to determine which data for a given species is the most appropriate for use in case work, and these validation studies should be conducted during each season.
8. Forensic entomologists should recognize that the estimates they provide as to when colonization of a body occurred are at best underestimates, because the data these estimates are based were developed under conditions that likely selected for an increased rate of development, and thus a forensic entomologist provides the minimum post mortem interval.

References

Anderson, G.S. 2000. Minimum and maximum developmental rates of some forensically important Calliphoridae (Diptera). Journal of Forensic Sciences 45: 824-832.

Ash, N. and B. Greenberg. 1975. Developmental temperature responses of the sibling species *Phaenicia sericata* and *Phaenicia pallescens*. Annals of the Entomological Society of America 68: 197-200.

Bartlett, A.C. 1985. Guidelines for genetic diversity in laboratory colony establishment and maintenance. In Handbook of Insect Rearing Vol. I. P. Singh and R.F. Moore, eds. Elsevier, Amsterdam, The Netherlands. 488 pp.

Baumgartner, D.L. 1993. Review of *Chrysomya rufifacies* (Diptera: Calliphoridae). Journal of Medical Entomology 30: 338-352.

Beck, S.D. Insect Photoperiodism. Second Edition. Academic Press, New York, NY. 387 pp.

Boatright, S.A. and J.K. Tomberlin. 2010. Effects of temperature and tissue type on the development of *Cochliomyia macellaria* (Diptera: Calliphoridae). Journal of Medical Entomology 47: 917-923.

Bravo, I.S.J. and F.S. Zucoloto. 1998. Performance and feeding behavior of *Ceratitis capitata*:
Comparison of a wild population and a laboratory population. Entomologia Experimentalis et Applicata 87: 67-72.

Buck, M. 2001. Protogyny, protandry, and bimodal emergence patterns in necrophagous Diptera. The Canadian Entomologist 133: 521-531.

Bush, G.L., R.W. Neck, and G.B. Kitto. 1976. Screwworm eradication: Inadvertent selection for noncompetitive ecotypes during mass rearing. Science 193: 491-493.

Byrd, J.H. and J.C. Allen. 2001. The development of the black blow fly, *Phormia regina* (Meigen). Forensic Science International 72: 79-88.

Byrd, J.H. and J.F. Butler. 1996. Effects of temperature on *Cochliomyia macellaria* (Diptera: Calliphoridae) development. Journal of Medical Entomology 33: 901-905.

Byrd, J.H. and J.F. Butler. 1997. Effects of temperature on *Chrysomya rufifacies* (Diptera: Calliphoridae) development. Journal of Medical Entomology 34: 353-358.

Byrd, J.H. and J.F. Butler. 1998. Effects of temperature on *Sarcophaga heamorrhoidalis* (Diptera: Sarcophagidae) development. Journal of Medical Entomology 35: 694-698.

Cammack, J.A. 2009. Effects of parasitism and soil compaction on pupation behavior of the green bottle fly Lucilia sericata (Meigen) (Diptera: Calliphoridae). MS Thesis, Clemson University, Clemson, SC, USA.

Catts, E.P. and M.L. Goff. 1992. Forensic entomology in criminal investigations. Annual Review of Entomology 37: 253-272.

Clark, K. L. Evans, and R. Wall. 2006. Growth rates of the blowfly, *Lucilia sericata*, on different body tissues. Forensic Science International 156: 145-149.

Colloff, M.J. 1987. Effects of temperature and relative humidity on development times and mortality of eggs from laboratory and wild populatiosn of the European Houst-Dust Mite *Dermatophagoides pteronyssinus* (Acari: Pyroglyphidae). Experimental and Applied Acarology 3: 279-289.
Davidowitz, G. L.J. D'Amico, and H.F. Nijhout. Critical weight in the development of insect body size. Evolution and Development 5: 188-197.

Erzinclioglu, Y.Z. 1985. The entomological investigation of a concealed corpse. Med Sci Law 25: 228-230.

Fagerström, T. and C. Wiklund. 1982. Why do males emerge before females? Protandry as a mating strategy in male and female butterflies. Oecologia 52: 164-166.

Florin, A.-B. and N. Gyllenstrand. 2002. Isolation and characterization of polymorphic microsatellite markers in the blowflies *Lucilia illustris* and *Lucilia sericata*. Molecular Ecology Notes 2: 113-116.

Foote, D. and J.R. Carey. 1987. Comparative demography of a laboratory and a wild strain of the oriental fruit fly, *Dacus dorsalis*. Entomologia Experimentalis et Applicata 44: 263-268.

Goff, M.L. 1991. Comparison of insect species associated with decomposing remains recovered inside dwellings and outdoors on the island of Oahu, Hawaii. Journal of Forensic Sciences 36: 748-753.

Goff, M.L. 1992. Problems in estimation of postmortem interval resulting from wrapping of the corpse: a case study from Hawaii. Journal of Agricultural Entomology 9: 237-243.

Grassberger, M. and C. Reiter. 2001. Effect of temperature on *Lucilia sericata* development (Diptera: Calliphoridae) development with special reference to the isomegalen- and isomorphen-diagram. Forensic Science International 120: 32-36.

Grassberger, M. and C. Reiter. 2002. Effect of temperature on development of the foresically important holarctic blow fly *Protophormia terraenovae* (Robineau-Desvoidy) (Diptera: Calliphoridae). Forensic Science International 128: 177-182.

Greenberg, B.L. 1991. Flies as forensic indicators. Journal of Medical Entomology 28: 565-577.

Hightower, B.G. G.E. Spates, Jr., and J.J. Garcia. 1972. Growht and critical size at pupation for larvae of the screwworm developing in fresh wounds. Journal of Economic Entomology 65: 1349-1352.

Hu, Y. X. Yuan, F. Zhu, and C. Lei. 2010. Development time and size-related traits in the oriental blowfly, *Chrysomya megacephala* along a latitudinal gradient from China. Journal of Thermal Biology 35: 366-371.

Ireland, S. and B. Turner. 2006. The effects of larval crowding and food type on the size and development of the blowfly, *Calliphora vomitoria*. Forensic Science International 159: 175-181.

Kamal, A.S. 1958. Comparative study of thirteen species of sarcosaprophagous Calliphoridae and Sarcophagidae (Diptera) I. Bionomics. Annals of the Entomological Society of America 51: 261-271.

Mackerras, M.J. 1933. Observaions on the life-histories, nutritional requirements and fecundity of blowflies. Bulletin of Entomological Research 24: 253-362.

Matsuura, K. 2006. Early emergence of males in the termite Reticulitermes speratus (Isoptera: Rhinotermididae): Protandry as a side effect of sexual size dimorphism. Annals of the Entomological Society of America 99: 625-628.

Nabity, P.D. L.G. Higley, and T.M. Heng-Moss. 2006. Effects of temperature on development of *Phormia regina* (Diptera: Calliphoridae) and use of developmental data in determining time intervals in forensic entomology. Journal of Medical Entomology 43: 1276-1286.

Nabity, P.D. L.G. Higley, and T.M. Heng-Moss. 2007. Light-induced variability in development of forensically important blow fly *Phormia regina* (Diptera: Calliphoridae). Journal of Medical Entomology 44: 351-358.

National Research Council Committee on Identifying the Needs of the Forensic Sciences Community; Committee on Applied and Theoretical Statistics. 2009. Strengthening forensic science in the United States: A path forward. National Academies Press, Washington, D.C. 352 pp.

Picard, C.J. and J.D. Wells. 2009. Survey of the genetic diversity of Phormia regina (Diptera: Calliphoridae) using amplified fragment length polymorphisms. Journal of Medical Entomology 46: 664-670.

Picard, C.J. and J.D. Wells. 2010. The population genetic structure of North American *Lucilia sericata* (Diptera: Calliphoridae), and the utility of genetic assignment methods for reconstruction of postmortem corpse relocation. Forensic Science International 195: 63-67.

Pujol-Luz J.R. P.A.D.C. Francez, A. Ururahy-Rodrigues, and R. Constantino. 2008. The black soldier-fly, *Hermetia illucens* (Diptera, Stratiomyidae), used to estimate the postmortem interval in a case in Amapa State, Brazil. Journal of Forensic Sciences 53: 476-478.

Ribeiro, C.d.S. and C.J. Von Zuben. 2010. Nutritional ecology of blowflies (Diptera, Calliphoridae): estimates of critical larval weight for pupation of two different diets. Revista Brasileira de Entomologia 54: 661-664.

Rivers, D.B. T. Ciarlo, M. Spelman, and R. Brogan. 2010. Changes in development and heat shock protein expression in two species of flies (Sarcophaga bullata [Diptera: Sarcophagidae] and Protophormia terraenovae [Diptera: Calliphoridae]) reared in different sized maggot masses. Journal of Medical Entomology 47: 577-589.

Saks, M.J. and J.J. Koehler. 2005. The coming paradigm shift in forensic identification science. Science 309: 892-895.

Saunders, D.S. and A. Bee. 1995. Effects of larval crowding on size and fecundity of the blow fly, *Calliphora vicina* (Diptera: Calliphoridae). European Journal of Entomology 92: 615-622.

Sharpe, P.J.H. and D.W. DeMichele. 1977. Reaction kinetics of poikilotherm development. Journal of Theoretical Biology 64: 649-670.

Tachibana, S.-I. and H. Numata. 2004. Effects of temperature and photoperiod on the termination of larval diapauses in *Lucilia sericata* (Diptera :Calliphoridae). Zoological Science 21: 197-202.

Tarone, A.M. and D.R. Foran. 2006. Components of developmental plasticity in a Michigan population of *Lucilia sericata* (Diptera: Calliphoridae). Journal of Medical Entomology 43: 1023-1033.

Thornhill, R. and J. Alcock. 1983. The evolution of insect mating systems. Harvard University Press, Cambridge, MA.

Tomberlin, J.K. R. Mohr, M.E. Benbow, A.M. Tarone, and S. VanLaerhoven. 2011. A roadmap for bridging basic and applied research in forensic entomology. Annual Review of Entomology 56: 401-421.

Tsiropolous, G.J. 1992. Feeding and dietary requirements of the tephritid fruit flies. In Advances in Insect Rearing for Research & Pest Management, T.E. Anderson and N.C. Leppla, eds. Westview Press, Boulder, CO. 515 pp.

Union-Tribune. 2002. Transcripts from the trial of David Westerfield. http://legacy.signonsandiego.com/news/metro/danielle/transcripts.html

VanLaerhoven, S.L. 2008. Blind validation of postmortem interval estimates using developmental rates of blow flies. Forensic Science International 180: 76-80.

Von Zuben, C.J. 1998. Individual oviposition behavior, percentage of egg hatching and minimum weight for successful pupation in populations of Chrysomya megacephala (F.). Annals of the Entomological Society of Brazil 27: 525-533.

Wells, J.D. and H. Kurahashi. 1994. *Chrysomya megacephala* (Fabricius) (Diptera: Calliphoridae) development: Rate, variation and the implications for forensic entomology. Japanese Journal of Sanitary Zoology 45: 303-309.

Wiklund, C., and C. Solbreck. 1982. Adaptive versus incidental explanations for the occurrence of protandry in a butterfly, *Leptidea sinapis* L. Evolution 36: 56-62.

Wilson, E.O. and W.H. Bossert. 1971. A primer of population biology. Sinauer Associated, Inc., Sunderland, Massachusetts, USA. 192 pp.

Yin, C.-M. and J.G. Stoffolano. 1998. Juvenile hormone regulation of reproduction in the cyclorrhaphous Diptera with emphasis on oogenesis. Archives of Insect Biochemistry and Physiology 35: 513-537.