ECOLOGY OF CALLIPHORID LARVAL MASSES AND POSTMORTEM COLONIZATION ESTIMATE VARIABILITY

By

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ABSTRACT

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The objectives of this work were to first, classify the insect and microbe interactions associated with carrion decomposition; and second, evaluate the variability in post-colonization interval (PCI) estimates among temperature sources and Calliphoridae species. Replicate (N = 6) swine carcasses were placed in a field and sampled regularly throughout decomposition. The ambient temperature at varying distances from the carcasses was recorded, and samples of larvae and their associated microbiomes were collected. Microbial communities were characterized using 16s amplicon sequencing. I hypothesized that insect and microbial community composition would shift over time and that different microbial communities would become more similar to each other over time. Additionally, I predicted that different Calliphoridae species would produce different accumulated degree hour (ADH) ranges and the closest temperature data set to the carcass would result in a more accurate PCI estimate. Larval masses consisted of three Calliphoridae species (*Cochliomyia macellaria, Lucilia coeruleiviridis,* and *Phormia regina*) whose relative abundances shifted over time. Microbial communities of the carcass surface, maggot mass, and internal larvae also changed over time as well as among the different locations. The closest temperature source produced the greatest ADH range but the estimated PCI was most similar to ADH ranges generated from local and regional weather station data. These findings are relevant for both carrion ecology and the forensic sciences by contributing to the understanding of the effect of carrion within ecosystems as well as advancing a new direction of forensic microbiology and the quantification of variability in forensic entomology.

To my parents, for never doubting my unusual interests and constantly providing unconditional love and support.

ACKNOWLEDGEMENTS

First and foremost, I would like to offer my sincerest gratitude to my committee. Thanks to my advisor, Dr. M. Eric Benbow, for his guidance and constant pushing towards new and challenging opportunities. It might not have always felt like peace and harmony at the time, but I am incredibly grateful for the experiences that have allowed me to learn and grow. Thanks to Dr. Jen Pechal for sharing her wisdom by always answering my questions and giving much needed advice. Thanks to Dr. Ruth Smith for taking the time to provide unique and valuable insight to my education. I could not have asked for better teachers and mentors.

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KEY TO ABBREVIATIONS

ADH Accumulated Degree Hours

CV Coefficient of Variation

PCI Post-Colonization Interval

PMI Post-Mortem Interval

SD Standard Deviation

SE Standard Error

CHAPTER 1: INTRODUCTION

Although carrion is defined as dead and decaying flesh, it is still full of life. Carrion is an important resource for a wide range of organisms (i.e., microbes, insects, and vertebrate scavengers) making it an important source of biodiversity and nutrients within an ecosystem (Barton et al. 2013a). Some of the most notable organisms that utilize carrion are the insects and microbes as they colonize the remains and are fundamental to the decomposition process (Payne 1965, Pechal et al. 2014a).

Necrophagous insect colonizers have been well studied due to their value in forensic investigations (Payne 1965, Byrd and Castner 2001, Kreitlow 2009, Benbow et al. 2013). Insects colonize in succession and the primary colonizers, blow flies (Diptera: Calliphoridae), will complete their life cycle with predictable development, depending primarily on temperature (Byrd and Castner 2001). Investigators are able to utilize the specific insects present and how developed they are at time of discovery, along with temperature history, to work backwards in order to estimate the post-colonization interval (PCI). Therefore, narrowing down time since death. There are many variables, however, within the decomposition process that can influence blow fly development and PCI estimates (Catts 1992). For example, insect development is highly dependent on temperature but investigators commonly rely on insect development data from previous studies conducted in laboratories and weather station data, which could be different than conditions and temperatures experienced at the scene (Byrd and Castner 2001, Catts and Haskell 1990, Baker et al. 1985, Catts 1992). The research presented here addresses the variability in blow fly development on swine carrion and PCI estimates from different temperature sources. Forensic entomology is a well-studied field but, nonetheless, there are

many variables to consider and a call to improve standards (National Research Council 2009). These results provide an important step towards developing necessary error rates for the analysis of entomological evidence.

Insects are not the only postmortem colonizers, however. Without insects, decomposition is slowed but does not cease due to microbes (Payne 1965, Pechal et al. 2014a). Recently much research has focused on the postmortem microbiome and its forensic implications. These microbial communities have the potential to assist with time since death estimates as they shift over time in a predictable manner, similar to insect succession (Pechal et al. 2013, 2014b, Metcalf et al. 2013, 2016). Since postmortem microbes and insects occur simultaneously (Pechal and Benbow 2016), understanding how these communities interact, and therefore gaining a greater knowledge the necrobiome (Benbow et al. 2013), is important for developing effective forensic techniques as well as understanding the effects of carrion on an ecosystem. Carrion is considered an ephemeral resource as organisms can break it down rapidly (Byrd and Castner 2001, Chaloner et al. 2002). Yet, carrion can have long lasting and widespread effects as its components are reincorporated into numerous levels of the food web (Benbow et al. 2015, Barton et al. 2013a, 2013b, Parmenter and MacMahon 2009). This research addresses the insect and microbe interactions occurring throughout swine decomposition by characterizing these two communities in order to determine how they change in relation to decomposition time and each other. These findings are significant for understanding carrion ecology, the advancement of a new direction of forensic microbiology, and the refinement of evidence collection and analysis.

CHAPTER 2: THE DYNAMIC MAGGOT MASS MICROBIOME

Abstract

Necrophagous insect studies have shown that decomposing vertebrate remains are an important ephemeral resource within an ecosystem. However, the microbes (e.g., bacteria and archaea) that were a part of the once living organism and the exogenous taxa that colonize this postmortem resource remain largely underexplored. Also, it is not well understood how these domains (i.e., microbes and insects) interact to recycle decaying biomass, an important mechanistic question for ecosystem function ecology. To better understand the microbial community dynamics throughout decomposition, we used replicate swine carcasses (N = 6) as models for mammalian postmortem decomposition to characterize epinecrotic microbial communities from: the abdominal skin of replicate carcasses; the internal microbiome of individual necrophagous dipteran larvae (maggots); and the microbiome of dipteran larval masses that had colonized the carcasses. Blow flies (Diptera: Calliphoridae) were the targeted insect taxon, as these are primary colonizers of vertebrate carcasses. Sampling occurred every twelve hours for the duration of the decomposition process. We characterized these microbial communities over time using high-throughput 16S amplicon sequencing. The relative abundance of microbial taxa changed over decomposition (temporal shift) as well as across sampling locations (spatial shift) suggesting significant interactions between the environment, microbes, and insect larvae. Maggot masses were represented by multiple blow fly species in each mass: *Phormia regina* (Meigen), *Lucilia coeruleiviridis* (Macquart), and *Cochliomyia macellaria* (Fabricius). Relative abundance of these species within the larval mass also changed as decomposition progressed suggesting the presence of certain Calliphoridae species within a mass

may be associated with temporal shifts of the microbial communities. These results provide new insight into the community ecology of carrion decomposition by providing new data on the interactions of microbes and dipteran larvae over time.

Introduction

Death is the beginning of new life within an ecosystem. After an animal dies, a wide range of organisms (i.e., microbes, insects, and vertebrate scavengers) will begin to decompose the carcass, utilize it as a food and/or mating resource, and reincorporate its constituents (e.g., nitrogen) into the ecosystem. All of these organisms coming together create a complex system of interdomain interactions that are collectively described as the necrobiome (Benbow et al. 2013). Although carrion is an ephemeral resource, members of the necrobiome become integrated into food webs and ecosystems with potentially far reaching and lasting effects on ecosystem structure and function (Benbow et al. 2015b, Barton et al. 2013a, 2013b, Parmenter and MacMahon 2009). Despite the significant impacts of carrion decomposition on an ecosystem it remains a largely understudied area of ecology.

One aspect of carrion ecology that has received considerable attention, however, is the succession of postmortem arthropod colonizers, specifically the blow flies (Diptera: Calliphoridae) (Payne 1965, Byrd and Castner 2001, Kreitlow 2009, Benbow et al. 2013). Arriving shortly after death, blow flies search for suitable habitat or substrate in which to reproduce, feed, and provide a food resource for their offspring (Byrd and Castner 2001). Their high fecundity and growth rates result in an efficient conversion of the carrion resource into insect biomass (Byrd and Castner 2001, Chaloner et al. 2002). This conversion efficiency is also evidenced in the growing interest in using saprophagous dipteran larvae to manage food and other organic waste, such as manure (Pastor et al. 2015, Nguyen et al. 2015). Without blow flies and other scavengers, both vertebrate and invertebrate, the turnover rate of carrion back into the ecosystem would be greatly reduced (Payne 1965, Pechal et al. 2014a). Nevertheless, even this well-studied area of carrion ecology has neglected an important component, the interaction of

blow flies with the microbes (e.g., bacteria and archaea) of the decaying organic matter (Pechal et al. 2013, Pechal et al. 2014). Indeed, studies of these interactions are beginning to demonstrate that microbes can play an important role in the attraction behavior of carrion flies to resources (Zheng et al. 2013, Ma et al. 2012, Jordan et al. 2015), suggesting additional interactions are likely important to the ecology of carrion within ecosystems.

Microbial communities are not only present throughout carrion decomposition but also have a predictable structural (community composition) and functional change over time (Pechal et al. 2013, 2014b, Metcalf et al. 2013, 2016), which reflects changes within entomological communities (Pechal et al. 2014b). Microbial communities are present within and on blow fly larvae as well as on the surface of the carrion itself (Pechal and Benbow 2016), but how these different communities interact over the course of decomposition is relatively unknown. In order to more fully understand these interdomain interactions of carrion ecology, we identified the calliphorid larval community of decomposing carrion, as well as characterized the associated microbial communities of the carcass surface, the internal larval microbiome, and the larval (maggot) mass microbiome throughout swine carcass decomposition. We hypothesized that the relative abundance of certain microbial taxa in each sampling area would shift over time, and that taxa would become more or less abundant among the interacting communities of the skin, larvae, and larval masses. We predicted that the internal larval microbiome would be, in part, determined from their external environment and food source (i.e., the carcass). Therefore, we expected the internal and external microbiomes of the larvae to have increasingly similar microbial community compositions as decomposition progressed. We also hypothesized there would be a detectable sequence of insect species colonizers that would change as the carcasses

decomposed, which would be consistent with previous studies on postmortem insect succession (Payne 1965, Byrd and Castner 2001, Kreitlow 2009, Benbow et al. 2013).

Materials and Methods

Study Site

The study was conducted from 19-27 August 2014 at Purdue University's Forensic Entomology Research Compound in West Lafayette, Indiana (40°25'36.0"N, 86°56'57.0"W). Six (three male and three female) swine carcasses (*Sus scrofa* L.), averaging 19.65 kg (± 5.44 SD) and approximately 80.0 cm in length, were purchased from a local farm and therefore exempt from university IACUC review. Carcasses were immediately placed in plastic bags during transport to the site (approximately 4 h), as previously described (Benbow et al. 2013, Pechal et al. 2013, Pechal et al. 2014a, Pechal et al. 2014b). The carcasses were placed on two East-West running transects approximately 50 m apart in a grassy field surrounded by deciduous forest (Fig. 1). All carcasses were oriented on their left side with heads directed north and placed on top of stainless steel grates (~1.0 m^2), with ~5.0 cm^2 openings, located on the ground to allow for lifting the individual carcasses during sample collection. Cages (~1.0 m^3) constructed of chicken mesh wire and treated lumber were secured over the carcasses and the grates to prevent vertebrate scavenging (Fig. 2). Microbial and insect communities were sampled every 12 h at approximately 0700 h and 1900 h for 8 consecutive days, which was the amount of time for the carcasses to fully decompose to only bones and skin, and dipteran larvae to develop and begin the post-feeding dispersal from the resources for pupation.

In order to calculate accumulated degree hours (ADH) at a microscale, which are important for comparing rates of larval development of different populations, one HOBO™ temperature logger (Onset Computer Corporation, Bourne, MA, USA) was attached to the east side of each anti-vertebrate scavenging cage, approximately 0.90 m off the ground. Ambient

Figure 1: Placement of swine carcasses in the field.

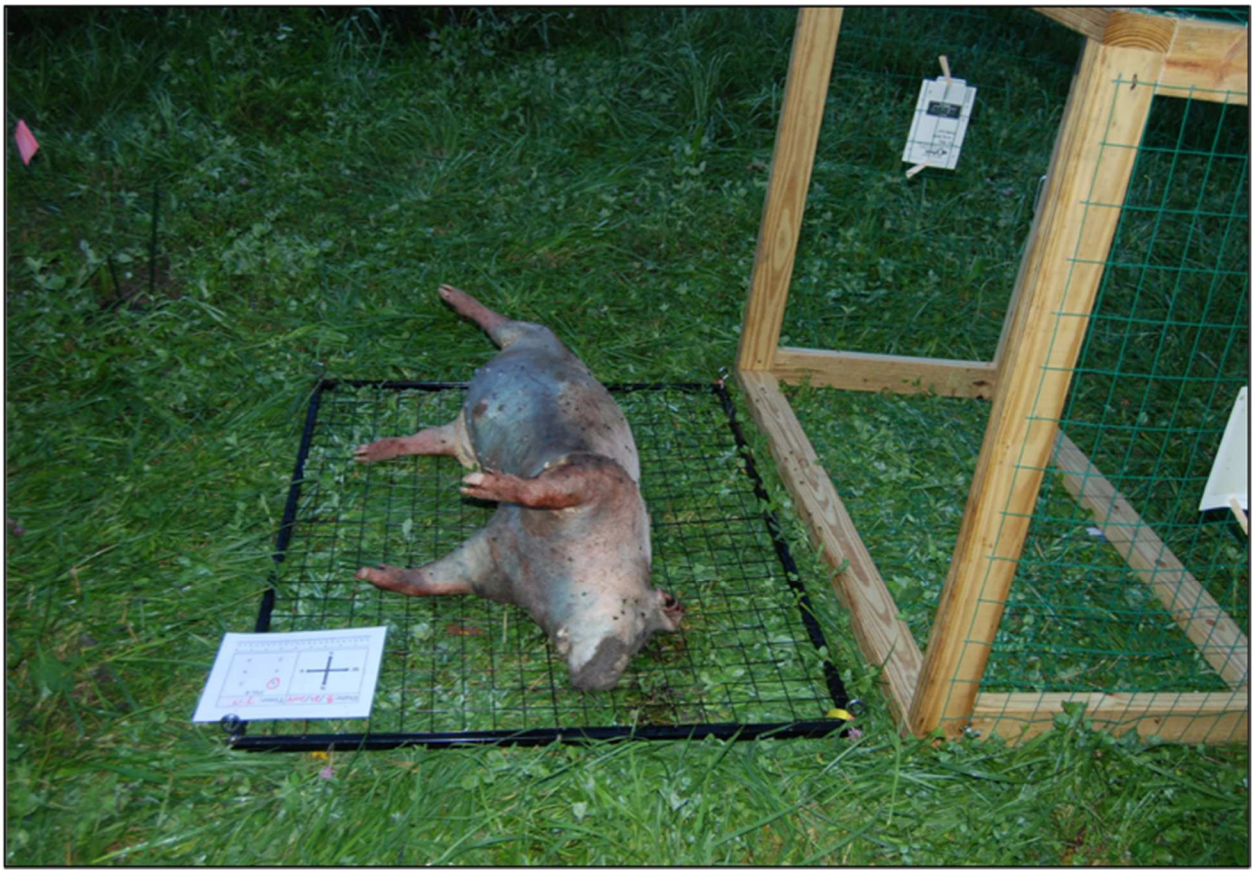

Figure 2: Example of a swine carcass on the metal grate and an anti-vertebrate scavenging cage. (Photo credit: Elizabeth Went)

temperature (°C) was recorded every fifteen minutes throughout the duration of the study. An infrared thermometer (One World Technologies, Inc., Anderson, SC, USA) was used to measure surface temperatures for the carcasses, blow fly larval masses, and the ground approximately 1 m away from each carcass at each 12 h sampling time period; the temperatures of three different locations for each substrate (e.g., the carcass or maggot mass) were recorded and then averaged. Temperature data were converted into ADH using thermal summation models with a base temperature of 0 °C (Pechal et al. 2014b, VanLaerhoven 2008). Multiple rain and thunderstorm events occurred throughout this study, prohibiting and delaying a few sampling time points. Data from the Purdue University Airport (40°24'48.5"N, 86°56'26.1"W) weather station, located 1.63 km southeast of the study site, were used to assess rainfall over the course of this study (Fig. 3).

Microbe and Insect Sampling

The epinecrotic microbial community of the skin (i.e., external microbiome) for each carcass was aseptically collected every 12 h by swabbing a single transect (posterior to anterior orientation) along the abdominal skin with a 15 cm sterile DNA-free cotton-tipped swab (Puritan Medical Products, Guilford, ME, USA), as described in Pechal et al. (2014b). The transect consisted of three combined areas (approximately 2.54 x 7.62 cm each) swabbed for 30 s each and care was taken not to resample the same areas throughout decomposition (Fig. 4). All swabs were stored at -20 °C until DNA isolation. Skin samples were no longer collected once maggot masses expanded to cover the sampling region.

In addition to the external microbiome of the carcass, the internal larval microbiome (microbes within the larvae) and the microbial communities of the larval mass (external microbes of the larval aggregate, the maggot mass microbiome) were also characterized. At each

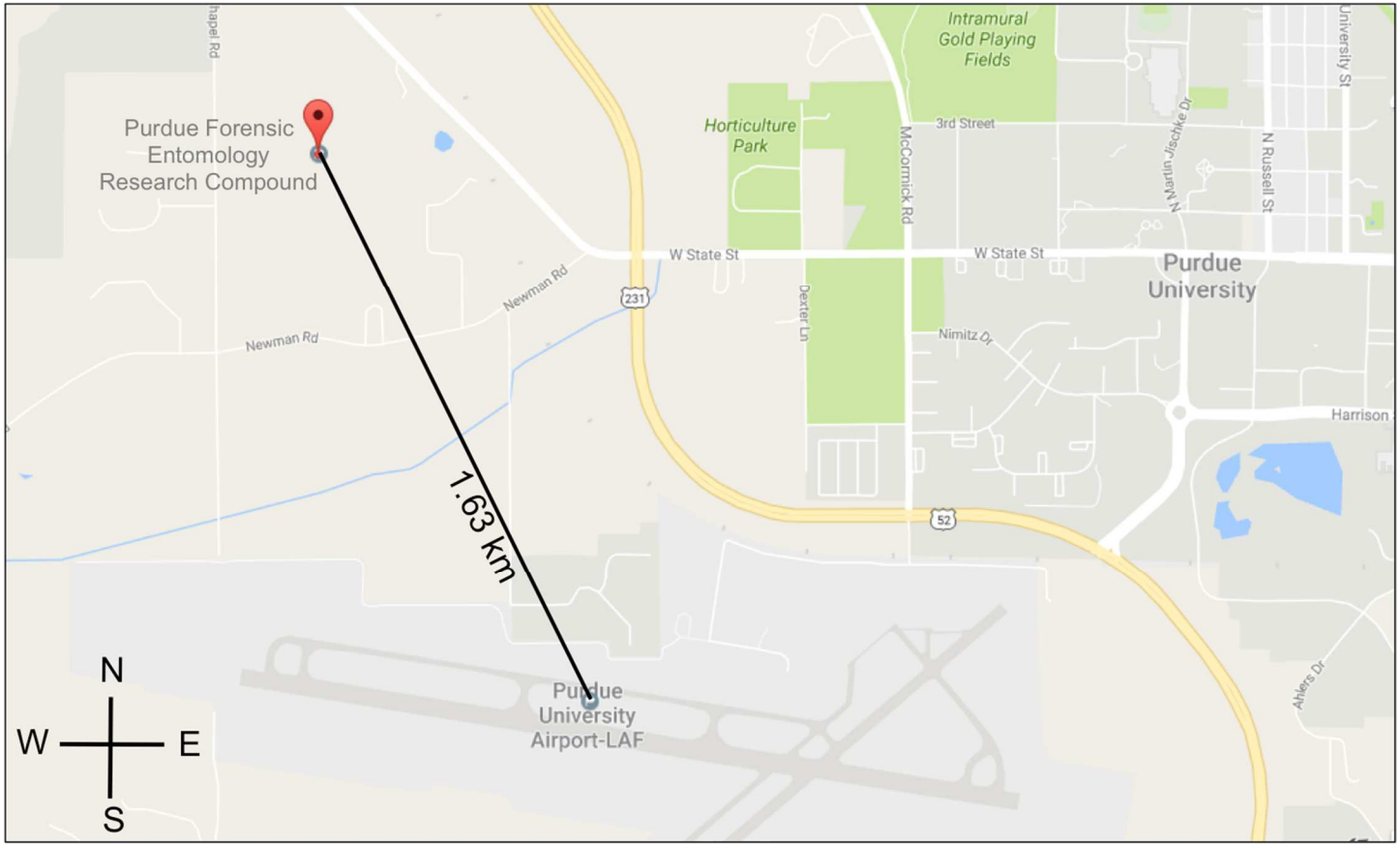

Figure 3: Map showing the location of the Purdue Forensic Entomology Research Compound and the Purdue University Airport in relation to each other and Purdue University.

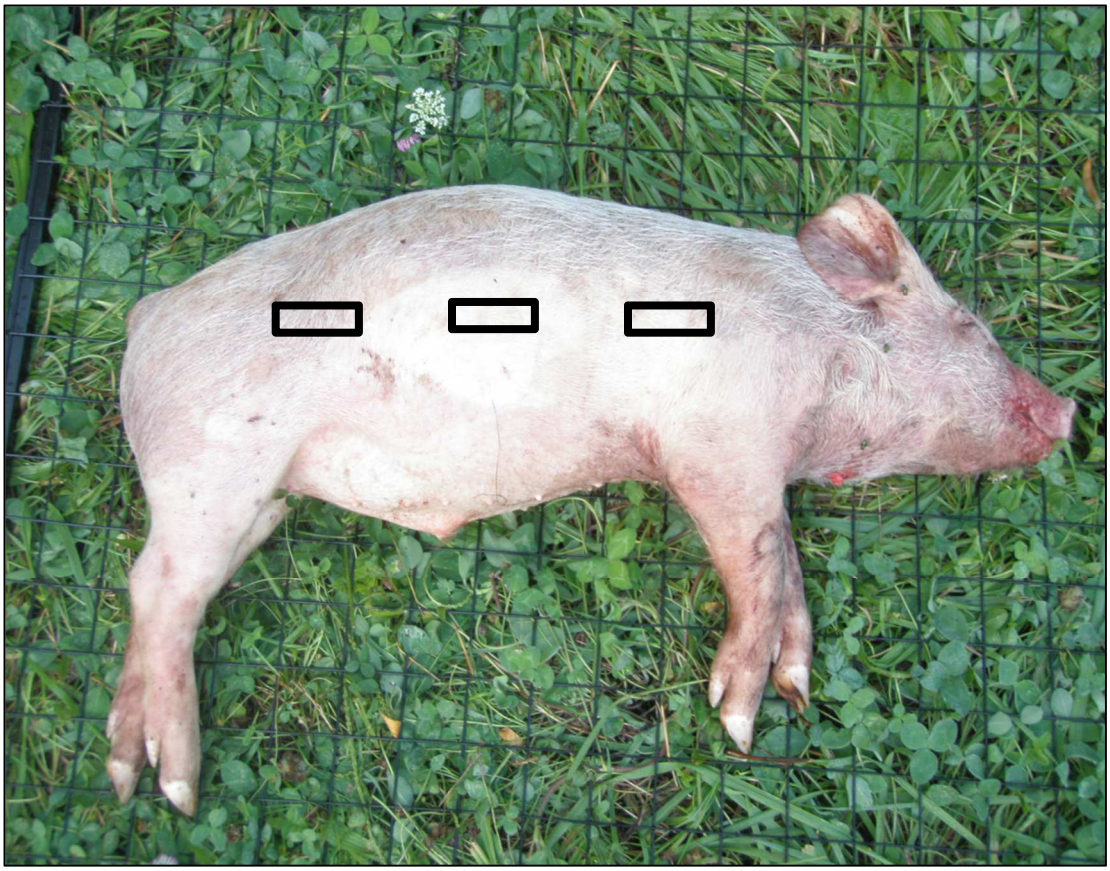

Figure 4: Example sampling region of the carcass epinecrotic skin microbiome. Black rectangles indicate the swabbed region: three areas combined for an abdominal transect.

sampling period, approximately 20-30 blow fly larvae were collected using a sterile DNA-free cotton-tipped swab from three different areas of a single larval mass located on the carcass, and stored at -20 °C. Third instars were stored in 100% molecular grade ethanol and then identified to species level (Stojanovich et al. 1962, Wells et al. 1999). Three third instar specimens of the most abundant species collected, *Phormia regina* (Meigen) (Diptera: Calliphoridae), from each carcass at each sampling time point were used for internal microbiome analysis. The maggot mass microbiome was sampled every 12 h, for each mass present during decomposition, using a single sterile DNA-free cotton-tipped swab placed into three different areas of a single larval mass. Resulting samples were stored at -20 °C until DNA isolation.

DNA Isolation and Targeted Amplicon Sequencing

Samples from seven time points (0, 593, 1557, 1845, 2469, 3384, and 3962 ADH or 0, 24, 60, 72, 96, 132, and 156 h after carcass placement, respectively) were selected for microbial analysis due to sample quantity and progression of decomposition; all phases of decomposition were represented in the microbial analysis (Table 1). Time point 0 represented when the carcasses were placed in the field and insects were allowed access at approximately 4 h postmortem. Genomic DNA was isolated using the PureLink Genomic DNA Mini Kit (Grand Island, NY, USA) according to the manufacturer's protocol with the modification of adding 15µl lysozyme (15 mg/ml) during the lysis procedure to improve microbial cell lysis. Skin and maggot mass microbiome swab samples were extracted individually while larval specimens were surface sterilized and pooled (N = 3) for characterization of the internal larval microbiome (Pechal and Benbow 2016). For surface sterilization, specimens were rinsed in 10% bleach followed by three rinses in sterile deionized water (Pechal and Benbow 2016). DNA was

Table 1: Accumulated degree hours (ADH) with corresponding hours since carcass placement and dates. Temperature data were converted into ADH using thermal summation models with a base temperature of 0 °C (Pechal et al. 2014b, VanLaerhoven 2008).

ADH	Hours	Date	AM/PM
0	0	8/19/14	PM
593	24	8/20/14	PM
1557	60	8/22/14	AM
1845	72	8/22/14	PM
2469	96	8/23/14	PM
3384	132	8/25/14	AM
3962	156	8/26/14	AM

quantified using the Quant-iT dsDNA HS Assay kit and a Qubit 2.0 (Grand Island, NY, USA). Samples were sequenced at the Michigan State University Genomics Core facility (East Lansing, MI, USA) using Illumina MiSeq with amplification of the variable region 4 (V4) of the 16S rRNA gene using 515F/806R primers (5′-GTGCCAGCMGCCGCGG-3′, 5′-TACNVGGGTATCTAATCC-3′) (Claesson et al. 2010, Caporaso et al. 2011, Caporaso et al. 2012, Pechal and Benbow 2016). Raw sequencing data was assembled, quality-filtered, demultiplexed, and analyzed using the default settings in QIIME 1.9.0 (Caporaso et al. 2010) and consistent with Pechal and Benbow (2016). The relative abundance at the phyletic and family levels, or the microbial community profiles, was determined and analyzed over decomposition time and between locational communities. All sequencing data files were made available to the European Nucleotide Archive database as part of study numbers PRJEB18437, PRJEB18438, and PRJEB18439 (http://www.ebi.ac.uk/ena/data/view/PRJEB18437, http://www.ebi.ac.uk/ena/data/view/PRJEB18438, http://www.ebi.ac.uk/ena/data/view/PRJEB18439).

Statistical Analysis

Analysis of variance (ANOVA) and a paired t-test were conducted using SAS Studio 3.5 (SAS Institute Inc., Cary, NC, USA) in order to determine potential significant differences in maggot mass and ambient temperatures. A Friedman test was performed in SAS to evaluate blow fly species relative abundance over decomposition time and a Kruskal Wallis test was performed to evaluate microbial taxa relative abundance between different sampling communities and over time. A Spearman Rank test was used to evaluate correlations in the relative abundance of the

predominant taxa among the microbial communities over decompostion. All p-values were considered significant with alpha at or below 0.05.

Results

Maggot Mass Communities

Maggot masses were first observed on the anterior region of all carcasses (primarily in the buccal and nasal cavities) as well as on the posterior region of three carcasses at 1557 ADH (60 h). By 2469 ADH (96 h) each mass had expanded and combined into one large mass that, by visual assessment, covered the majority, if not all, of each carcass's surface area (Fig. 5). All masses had dispersed by 3962 ADH (156 h) with the exception of carcass 1 (Table 2; Fig. 6). Maggot mass temperatures over the course of decomposition were not significantly different among carcasses ($F = 0.26$, $df = 5$, $p = 0.931$), but were significantly different ($t = 16.45$, $p = < 0.0001$) from the ambient air temperature which averaged 9.29 °C (± 0.565 SE) lower than larval masses (Fig. 7).

Identification of third instar diptera collected from masses at five time points (1557, 1845, 2469, 3384, 3962 ADH) revealed multiple species were present within each mass and their relative abundances changed over time. Of the 643 larval specimens collected, three species were identified: *P. regina, Cochliomyia macellaria* (Fabricius) (Diptera: Calliphoridae), and *Lucilia coeruleiviridis* (Macquart) (Diptera: Calliphoridae). During the formation of the initial maggot masses (by 1557 ADH), *C. macellaria* was the most predominant species (52.0 ± 6.19% SE) on four carcasses, but as decomposition progressed, it was replaced by *P. regina* as the most predominant species (reaching 85.3 ± 4.53% SE) for all carcasses. The relative abundance of *L. coeruleiviridis* remained low (reaching 22.3 ± 9.59% SE) throughout decomposition (Fig. 8). Changes in relative abundance throughout decomposition were significant for *C. macellaria*

Figure 5: Example of a larval mass that expanded to cover the entirety of the carcass.

Table 2: Maggot mass locations, mean mass temperature, and ambient temperature for each carcass throughout decomposition. Gray areas represent times when maggot masses were no longer present.

Carcass	1			2			3			4			5			6			Mean	
ADH	Mass Location(s)	Mean Maggot Mass Temperature (C°)	Ambient Temperature (C°)	Mass Location(s)	Mean Maggot Mass Temperature (C°)	Ambient Temperature (C°)	Mass Location(s)	Mean Maggot Mass Temperature (C°)	Ambient Temperature (C°)	Mass Location(s)	Mean Maggot Mass Temperature (C°)	Ambient Temperature (C°)	Mass Location(s)	Mean Maggot Mass Temperature (C°)	Ambient Temperature (C°)	Mass Location(s)	Mean Maggot Mass Temperature (C°)	Ambient Temperature (C°)	Maggot Mass Temperature (C°)	Ambient Temperature (C°)
1557	Anterior Posterior	34.14	22.37	Anterior	38.73	26.99	Anterior Abdomen Posterior	30.70	24.85	Anterior	38.70	30.55	Anterior	37.30	30.22	Anterior Posterior	36.50	30.72	36.57	27.61
1845	Anterior Abdomen Posterior	35.13	26.35	Carcass	38.63	25.77	Anterior Posterior	37.23	26.99	Carcass	39.53	23.23	Anterior Posterior	38.83	23.40	Carcass	38.70	25.43	38.01	25.20
2469	Carcass	28.23	22.18	Carcass	28.47	22.59	Carcass	28.90	22.63	Carcass	27.80	22.63	Carcass	28.53	22.23	Carcass	31.83	22.25	28.96	22.42
3384	Anterior	35.83	20.79							Abdomen	37.40	21.75	Anterior	29.13	20.72				34.12	20.94
3962	Posterior	28.87	18.44																28.87	18.44

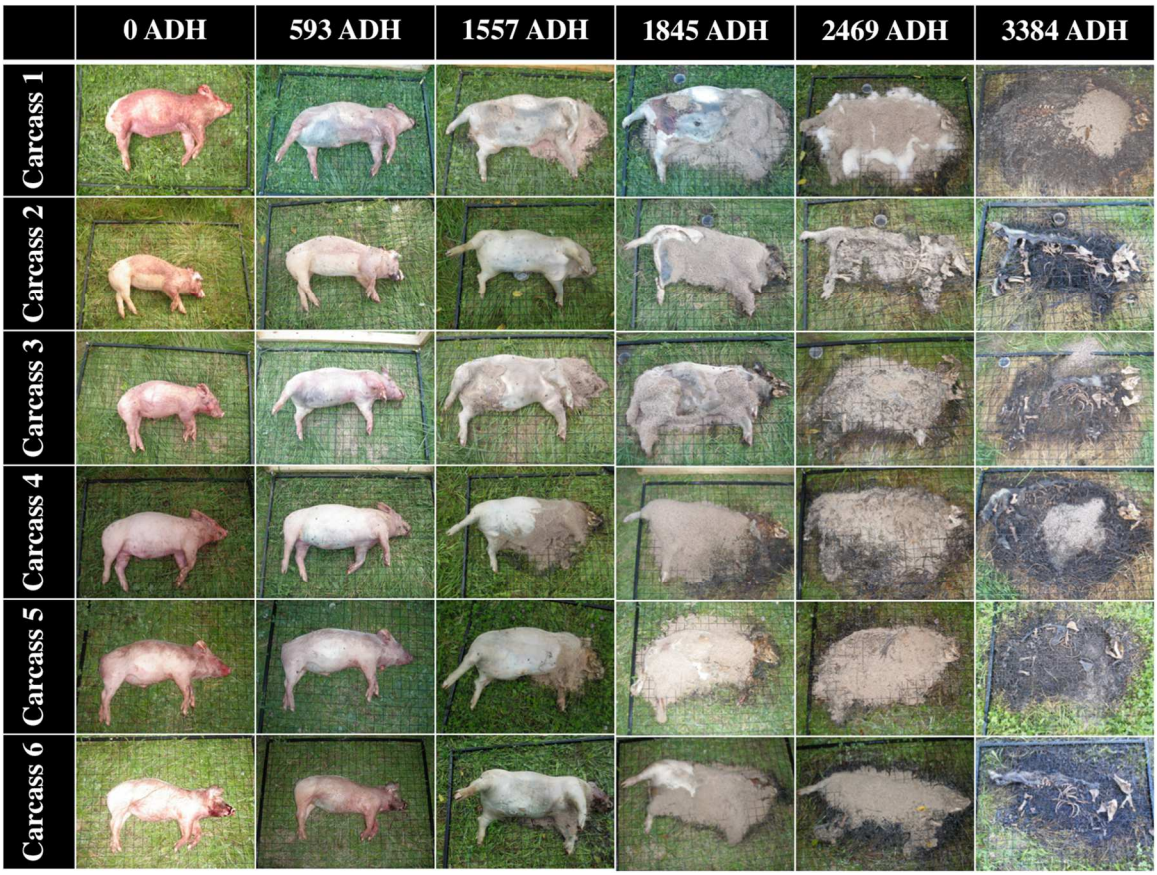

Figure 6: Taphonomic changes of individual carcasses at each sampling point over decomposition time expressed as accumulated degree hours (ADH). For scale, the average length of the carcasses was approximately 80.0 cm.

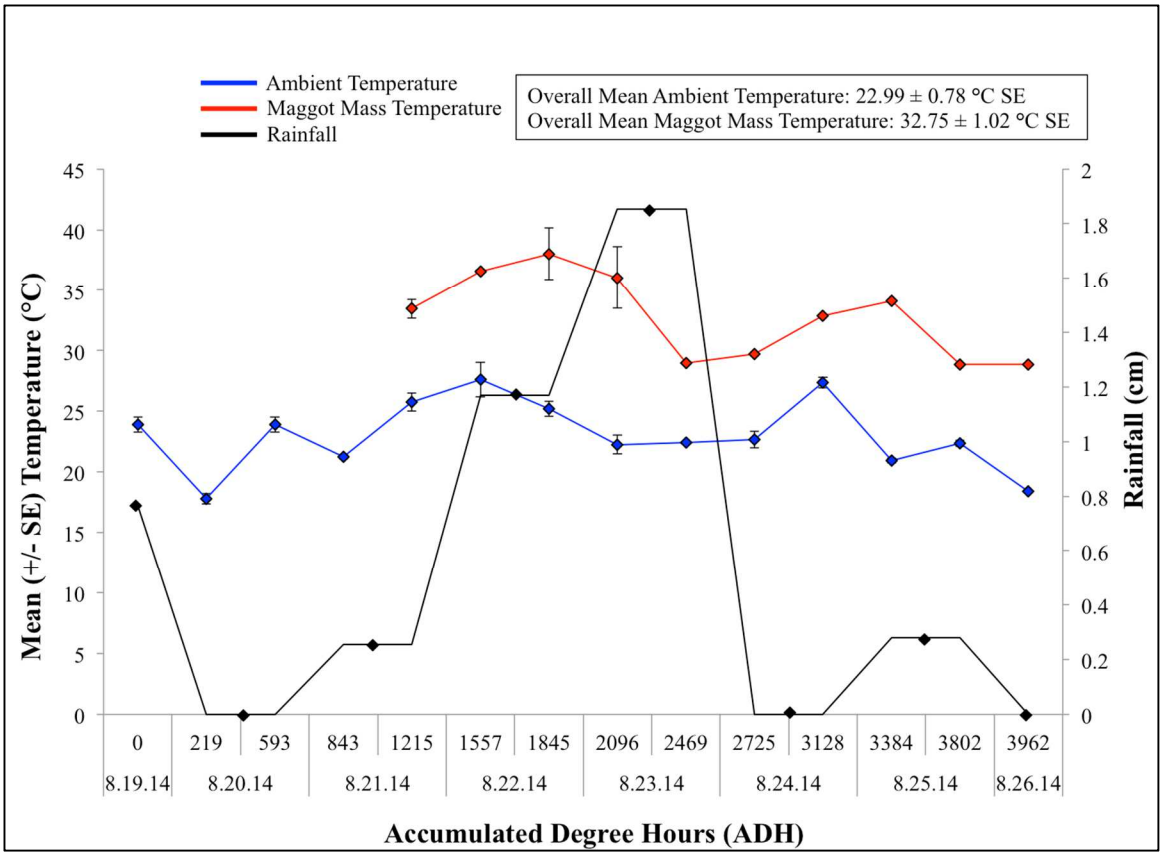

Figure 7: Rainfall and mean (SE) ambient and larval mass temperatures over decomposition time. The ADH are given above the calendar date along the x-axis. Rainfall was recorded daily while ambient and maggot mass temperatures were recorded for each carcass at each sampling point (every 12 h) and then averaged.

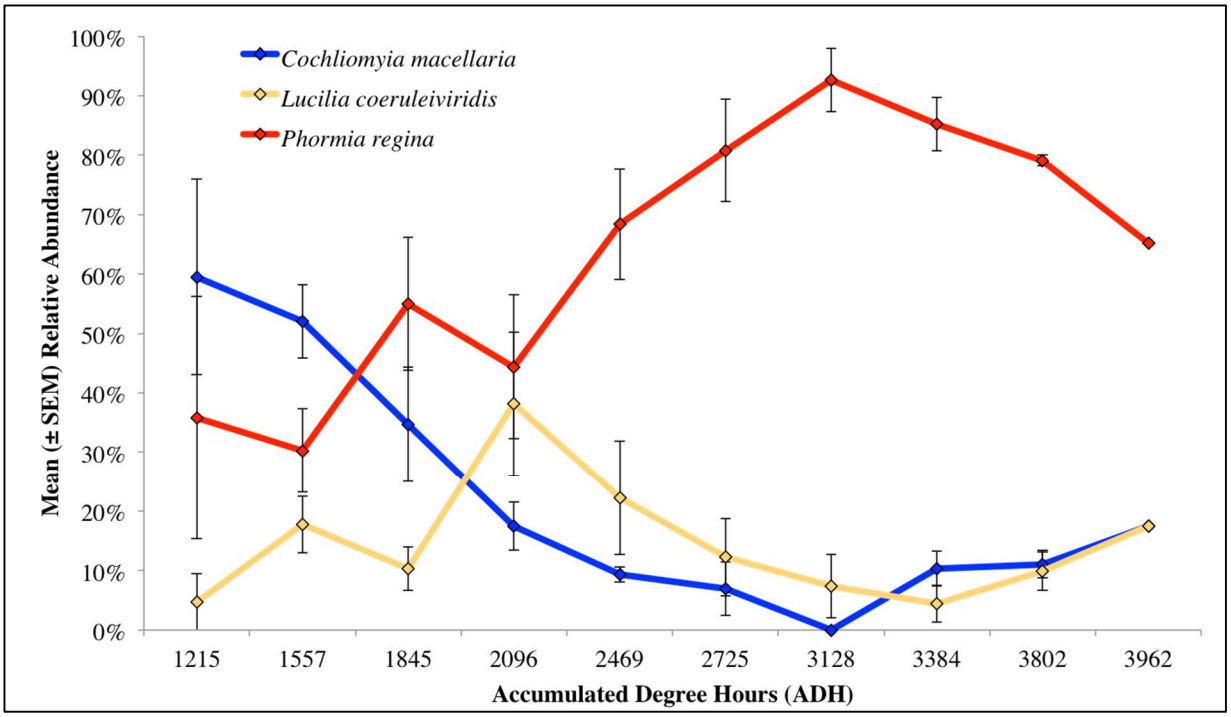

Figure 8: Mean (SE) relative abundance of third instar *Cochliomyia macellaria, Lucilia coeruleiviridis,* and *Phormia regina* over decomposition time expressed as accumulated degree hours (ADH).

(F = 4.40, df = 9, p = 0.008) and *P. regina* (F = 4.94, df = 9, p = 0.005), but not for *L. coeruleiviridis* (F = 1.22, df = 9, p = 0.360) (Table 3).

Microbial Community Composition

The predominant microbial phyla (>3% relative abundance) of the skin and maggot mass microbial communities were Proteobacteria, Firmicutes, and Bacteroidetes, while the internal larval microbiome was most represented by Proteobacteria and Firmicutes. For all three communities, the most prevalent class of Proteobacteria was Gammaproteobacteria. There was an inverse relationship between the relative abundance of Proteobacteria and Firmicutes over decomposition time, which was reflected in a Proteobacteria to Firmicutes (P/F) ratio that changed during decomposition from 0.38 P/F (0 ADH) to 1.02 P/F (1845 ADH) on the carcass surface, 1.01 P/F (1557 ADH) to 1.33 P/F (3962 ADH) in the maggot mass, and 0.91 P/F (1557 ADH) to 4.43 P/F (3962 ADH) for the internal larval microbiome. Early in the decomposition process Firmicutes relative abundance was higher (65.6 ± 6.44% SE) than Proteobacteria (25.0 ± 7.21% SE) on the carcass surface; however, as indicated by the P/F ratios, Firmicutes decreased while Proteobacteria increased over time. Near the end of decomposition (3962 ADH) the relative abundance of Firmicutes and Proteobactera was 35.3% and 47.1% in the maggot mass, respectively, while these phyla were 18.2% and 80.6% in the larval interal microbiome, respectively (Figs. 9 and 10).

There was a significant corrleation between Firmicutes and Proteobacteria relative abundance throughout decomposition for the carcass surface (ρ = -1.0, p = <0.0001) and the internal larval microbiome (ρ = -0.9, p = 0.037), but not for the maggot mass microbial communities (ρ = 0.8, p = 0.104). The relative abundances of Firmicutes and Proteobacteria were

Table 3: Calliphoridae species presence and relative abundance within individual maggot masses throughout decomposition.

ADH Range	Carcass	Maggot Mass Location	Species	n	Relative Abundance
1502-1618	Carcass 1	Anterior	*C. macellaria*	14	100.00%
		Posterior	*C. macellaria*	21	50.00%
			L. coeruleiviridis	1	2.38%
			P. regina	20	47.62%
	Carcass 2	Anterior	*C. macellaria*	16	51.61%
			L. coeruleiviridis	7	22.58%
			P. regina	8	25.81%
	Carcass 3	Anterior	*C. macellaria*	8	47.06%
			L. coeruleiviridis	4	23.53%
			P. regina	5	29.41%
		Abdomen	*C. macellaria*	5	55.56%
			P. regina	4	44.44%
		Posterior	*C. macellaria*	11	61.11%
			L. coeruleiviridis	1	5.56%
			P. regina	6	33.33%
	Carcass 4	Anterior	*C. macellaria*	7	36.84%
			L. coeruleiviridis	2	10.53%
			P. regina	10	52.63%
	Carcass 5	Anterior	*C. macellaria*	5	33.33%
			L. coeruleiviridis	5	33.33%
			P. regina	5	33.33%
	Carcass 6	Anterior	*C. macellaria*	5	55.56%
			L. coeruleiviridis	4	44.44%
		Posterior	*C. macellaria*	6	100.00%
1775-1903	Carcass 1	Anterior	*C. macellaria*	11	47.83%
			L. coeruleiviridis	1	4.35%
			P. regina	11	47.83%
		Abdomen	*C. macellaria*	13	92.86%
			L. coeruleiviridis	1	7.14%
		Posterior	*C. macellaria*	25	80.65%
			L. coeruleiviridis	3	9.68%
			P. regina	3	9.68%
	Carcass 2	Carcass	*C. macellaria*	14	56.00%
			L. coeruleiviridis	5	20.00%
			P. regina	6	24.00%
	Carcass 3	Anterior	*C. macellaria*	2	11.76%
			P. regina	15	88.24%
		Posterior	*C. macellaria*	6	19.35%
			L. coeruleiviridis	3	9.68%
			P. regina	22	70.97%
	Carcass 4	Carcass	*C. macellaria*	5	23.81%
			P. regina	16	76.19%
	Carcass 5	Anterior	*C. macellaria*	1	7.14%
			P. regina	13	92.86%
		Posterior	*C. macellaria*	4	18.18%
			L. coeruleiviridis	2	9.09%
			P. regina	16	72.73%
	Carcass 6	Carcass	*C. macellaria*	8	25.81%
			L. coeruleiviridis	7	22.58%
			P. regina	16	51.61%

Table 3 (cont'd)

2396-2576	Carcass 1	Carcass	*C. macellaria*	3	10.71%
			L. coeruleiviridis	5	17.86%
			P. regina	20	71.43%
	Carcass 2	Carcass	*C. macellaria*	2	6.06%
			L. coeruleiviridis	3	9.09%
			P. regina	28	84.85%
	Carcass 3	Carcass	*C. macellaria*	1	6.67%
			L. coeruleiviridis	2	13.33%
			P. regina	12	80.00%
	Carcass 4	Carcass	*C. macellaria*	1	6.67%
			L. coeruleiviridis	10	66.67%
			P. regina	4	26.67%
	Carcass 5	Carcass	*C. macellaria*	2	12.50%
			P. regina	14	87.50%
	Carcass 6	Carcass	*C. macellaria*	2	13.33%
			L. coeruleiviridis	4	26.67%
			P. regina	9	60.00%
3318-3542	Carcass 1	Carcass	*C. macellaria*	2	10.53%
			P. regina	17	89.47%
	Carcass 4	Carcass	*C. macellaria*	4	18.18%
			L. coeruleiviridis	1	4.55%
			P. regina	17	77.27%
	Carcass 5	Carcass	*C. macellaria*	1	4.00%
			P. regina	24	96.00%
	Carcass 6	Carcass	*C. macellaria*	2	8.70%
			L. coeruleiviridis	3	13.04%
			P. regina	18	78.26%
3962	Carcass 1	Carcass	*C. macellaria*	4	17.39%
			L. coeruleiviridis	4	17.39%
			P. regina	15	65.22%

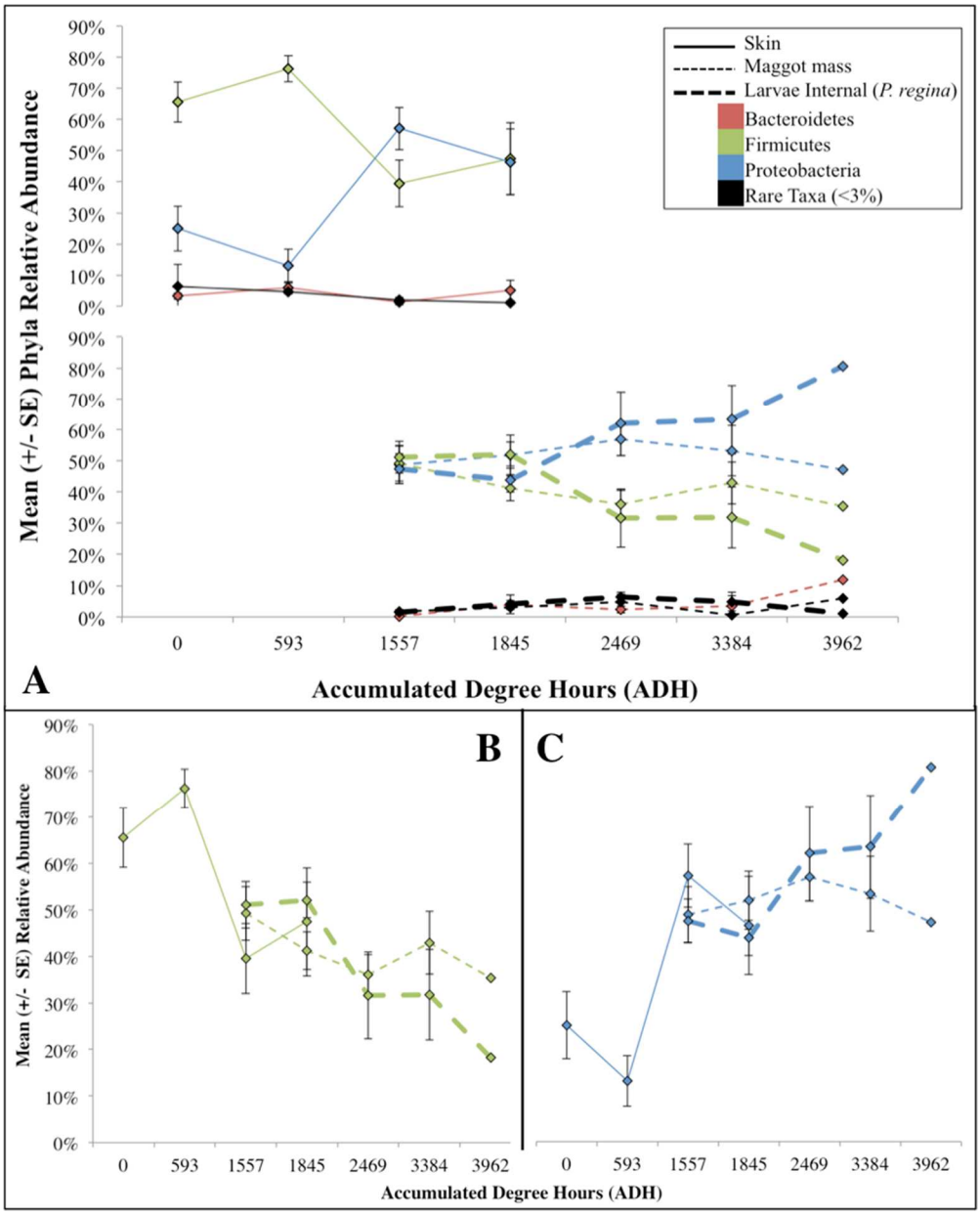

Figure 9: Mean (SE) relative abundance of dominant microbial phyla for carcass skin, maggot mass, and internal larval microbial communities over decomposition time expressed as accumulated degree hours (ADH). The top panel represents the skin microbial communities that were only present until 1845 ADH. The bottom panel shows the maggot mass microbial communities as well as those for *Phormia regina* larvae after the masses had formed (A). Mean (SE) relative abundance of Firmicutes (B) and Proteobacteria (C) over decomposition time for the carcass skin, maggot mass, and internal larval microbial communities.

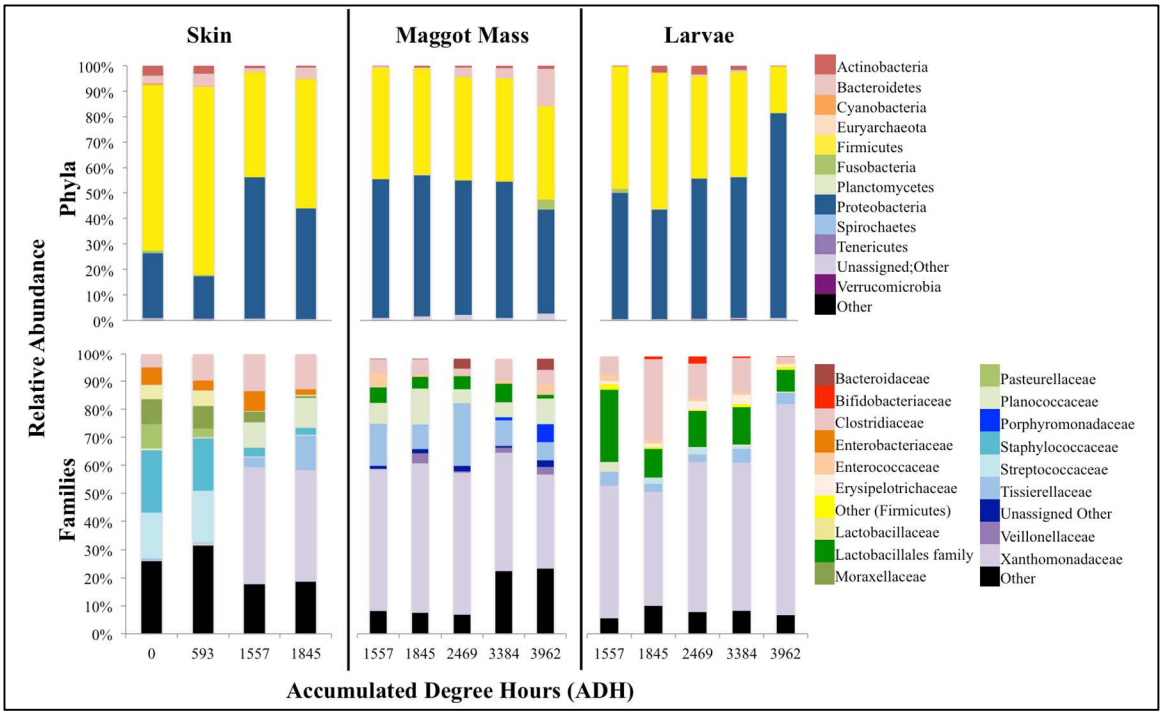

Figure 10: Relative abundance of the top ten most abundant microbial phyla (top panel) and families (bottom panel) for the carcass skin, maggot mass, and *Phormia regina* larvae microbial communities over decomposition time expressed as accumulated degree hours (ADH).

significantly different for the carcass surface (χ^2 = 9.46, df = 1, p = 0.002) and maggot mass (χ^2 = 5.62, df = 1, p = 0.018), but not for the internal larval microbiome (χ^2 = 3.53, df = 1, p = 0.060). For the predominant phyla (>3% overall relative abundance), between the maggot mass and internal larval communities there was a significant correlation over time of Proteobacteria (ρ = -0.9, p = 0.037), but not Firmicutes (ρ = 0.8, p = 0.104) and Bacteroidetes (ρ = -0.2, p = 0.747). Differences in relative abundance between the maggot mass and internal larval microbiome were not significantly different for any of the predominant phyla (Firmicutes: χ^2 = 0.061, df = 1, p = 0.805; Proteobacteria: χ^2 = 0.002, df = 1, p = 0.963; Bacteroidetes: χ^2 = 1.475, df = 1, p = 0.225). In addition, when maggot masses co-occurred with the skin communities (both sample locations were still present before the masses covered the entire carcass surface area) at 1557-1845 ADH, the relative abundances of dominant microbial phyla were similar across all three sampled communities: carcass surface, maggot mass, and larval internal. Ultimately, Proteobacteria abundance increased overall while Firmicutes decreased throughout decomposition in all of the microbial communities (Figs. 9 and 10; Table 4).

At the familial level, there were comparable results as the phyletic level, including an inverse relationship between the rare taxa (individual family relative abundances that were <3%) and Xanthomonadaceae. Immediately following carcass placement (0 ADH) skin microbial communities were dominated by rare taxa that collectively represented 39.80% (± 6.49 SE) of the community. At the same time (0 ADH), Xanthomonadaceae relative abundance was the lowest (0.22 ± 0.04% SE) until it increased at 1557 ADH (the same time maggot masses initially formed) to become the predominant family (41.7 ± 6.40% SE) of the skin communities. This increase in Xanthomonadaceae was also the case for the maggot mass and internal larval microbiomes, suggesting a mixing among these communities as decomposition progressed. For

Table 4: Presence and richness of microbial phyla throughout decomposition for each carcass in skin, maggot mass, and internal larval communities.

				PHYLA																															
ADH Range	Carcass	Location	Reads	Acidobacteria	Actinobacteria	Armatimonadetes	Bacteria, Other	Bacteroidetes	BRC1	Chlamydiae	Chlorobi	Chloroflexi	Crenarchaeota	Cyanobacteria	Elusimicrobia	Euryarchaeota	FBP	Fibrobacteres	Firmicutes	Fusobacteria	Gemmatimonadetes	Lentisphaerae	Nitrospirae	NKB19	Planctomycetes	Proteobacteria	Spirochaetes	Synergistetes	Tenericutes	[Thermi]	TM6	TM7	Unassigned, Other	Verrucomicrobia	WPS-2	WS3	WWE1	Richness
0	1	Skin	64,432	X	X			X		X		X		X		X			X	X					X	X	X		X				X	X				15
	2	Skin	79,588	X	X			X		X				X	X	X			X	X					X	X	X		X				X	X				15
	3	Skin	94,251	X	X	X		X		X		X		X		X			X	X					X	X	X		X			X	X	X				17
	4	Skin	75,348	X	X			X		X	X	X	X	X		X		X	X	X	X		X		X	X	X		X				X	X				20
	5	Skin	127,203	X	X		X	X		X		X	X	X		X			X	X	X				X	X	X		X	X			X	X				19
	6	Skin	79,011	X	X	X	X	X		X		X	X	X		X	X	X	X	X	X		X		X	X	X		X				X	X				22
546-	1	Skin	28,445		X			X						X		X			X			X				X	X		X				X	X				11
639	2	Skin	105,946	X	X		X	X		X				X		X	X		X	X					X	X	X		X				X	X				16
	3	Skin	67,647	X	X			X		X				X		X			X	X					X	X	X		X				X	X				14
	4	Skin	72,551	X	X		X	X		X		X		X		X		X	X	X			X		X	X	X	X	X				X	X				19
	5	Skin	96,499	X	X	X	X	X		X		X		X		X			X	X	X	X			X	X	X		X				X	X		X		20
	6	Skin	67,357	X	X			X		X		X		X	X	X	X	X	X	X					X	X	X		X	X			X	X				18
1502-	1	Skin	55,026	X	X		X	X		X				X		X			X	X					X	X			X				X	X	X			15
1618		Maggot Anterior	59,578		X			X											X	X					X	X							X					7
		Mass Posterior	67,364		X		X	X				X		X					X	X					X	X			X				X	X				12
		Larvae Posterior	90,149		X			X						X					X							X			X				X					7
	2	Skin	57,504		X			X						X		X			X						X	X			X				X	X				10
		Maggot Anterior Mass	44,895		X		X	X											X						X	X							X					7
		Larvae Anterior	88,159		X			X						X					X							X							X					6
	3	Skin	75,769		X			X						X					X	X						X			X				X					8
		Maggot Anterior	47,339		X			X											X	X						X			X				X					7
		Mass Abdomen	96,329		X									X		X			X	X					X	X							X	X				9
		Posterior	53,752		X			X						X					X	X						X							X	X				8
		Larvae Anterior	76,682		X		X	X		X		X		X					X	X					X	X							X	X				12
		Abdomen	79,567		X		X	X						X					X	X				X	X	X			X		X		X					12
		Posterior	84,303		X			X						X					X	X						X							X					7
	4	Skin	69,866		X		X	X						X		X			X	X					X	X			X				X					11
		Maggot Anterior Mass	69,927		X		X	X											X						X	X							X					7
		Larvae Anterior	65,348		X			X		X				X					X							X							X	X				8
	5	Skin	95,842		X			X						X		X			X							X							X					7
		Maggot Anterior Mass	77,994		X					X									X						X	X							X	X				7
		Larvae Anterior	108,924		X			X						X					X							X							X					6
	6	Skin	84,474		X		X	X					X	X		X			X	X					X	X	X						X					12
		Maggot Anterior	76,408	X	X			X						X					X							X							X	X				8
		Mass Posterior	121,160		X			X											X	X						X			X				X					7
1775-	1	Skin	41,752		X			X						X					X							X			X				X					7
1903		Maggot Anterior	22,016		X			X											X	X						X							X					6
		Mass Abdomen	75,760		X		X	X						X					X						X	X		X	X				X					10
		Posterior	16,666	X	X			X				X							X	X						X	X						X	X				10
		Larvae Anterior	74,843		X	X		X		X		X		X					X	X					X	X			X			X	X	X				14
		Posterior	75,480		X			X	X			X							X						X	X			X				X	X				10
	2	Skin	102,118		X			X		X				X		X		X	X	X		X				X			X				X	X				13
		Maggot Mass	51,696		X		X	X						X		X			X		X				X	X							X	X				11
		Larvae	91,348		X			X				X	X	X		X			X	X					X	X							X	X				12
	3	Skin	17,511	X	X			X								X			X	X						X			X				X					9
		Maggot Anterior	36,476		X						X								X	X						X							X					6
		Mass Posterior	67,489		X			X					X						X	X					X	X			X				X					9
		Larvae Anterior	108,588	X	X			X								X			X	X					X	X			X				X					10
		Posterior	135,866		X	X	X	X		X		X	X	X		X			X	X		X			X	X			X			X	X	X				18
	4	Maggot Mass	4,992																X							X							X					3
		Larvae	79,667		X		X	X				X		X					X						X	X			X		X		X	X				12
	5	Maggot Anterior	55,453		X			X		X		X							X	X					X	X							X	X				10
		Mass Posterior	66,472		X			X						X					X	X					X	X			X				X	X				10
		Larvae Anterior	118,144		X			X				X		X					X	X					X	X							X					9
		Posterior	107,297		X		X	X		X		X	X	X					X						X	X			X				X					12
	6	Skin	24,588		X			X						X					X							X			X				X	X				8
		Maggot Mass	810		X			X											X							X												4
		Larvae	100,086		X			X						X		X			X	X			X		X	X			X				X	X				12
2396-	1	Maggot Mass	65,240		X		X	X						X					X	X					X	X			X				X					10
2576		Larvae	115,686		X			X	X			X		X					X				X		X	X			X				X	X				12
	2	Maggot Mass	358					X											X							X												3
		Larvae	6,888		X			X											X						X	X							X					6
	3	Maggot Mass	260																X	X						X												3
		Larvae	40,604	X	X			X				X		X		X			X						X	X	X		X				X	X				13
	4	Maggot Mass	212		X														X							X												3
		Larvae	440					X											X							X												3
	5	Maggot Mass	235					X											X							X												3
		Larvae	66,176		X			X											X						X	X							X	X				7
	6	Maggot Mass	28,682	X	X			X				X		X					X	X					X	X							X	X				11
		Larvae	23,176		X			X						X		X		X	X				X			X	X							X	X				11
3318-	1	Maggot Mass	584		X			X											X	X						X												5
3542		Larvae	30,356		X			X											X		X				X	X	X		X				X	X				10
	4	Maggot Mas	329																X							X												2
		Larvae	75,740	X	X		X	X		X		X	X	X				X	X				X		X	X	X		X			X	X	X			X	19
	5	Maggot Mass	553					X											X							X							X					4
		Larvae	69,118		X			X		X									X						X	X							X	X				8
	6	Larvae	239					X											X							X												3
3962	1	Maggot Mass	337					X											X	X						X												4
		Larvae	65,419		X		X	X		X									X						X	X							X	X				9

the predominant families (>3% overall relative abundance), there was a significant correlation of a Lactobacillales family ($\rho = 0.9$, $p = 0.037$) between the maggot mass and internal larval communities, but not for Xanthomonadaceae ($\rho = -0.7$, $p = 0.188$), Tissierellaceae ($\rho = -0.1$, $p = 0.873$), Clostridiaceae ($\rho = -0.3$, $p = 0.624$), and Planococcaceae ($\rho = 0.1$, $p = 0.873$). However, relative abundances between the two communities were significantly different for all predominant families except Xanthomonadaceae (Clostridiaceae: $\chi^2 = 8.61$, $df = 1$, $p = 0.003$; Lactobacillales family: $\chi^2 = 6.02$, $df = 1$, $p = 0.014$; Planococcaceae; $\chi^2 = 18.9$, $df = 1$, $p = <0.0001$; Tissierellaceae: $\chi^2 = 5.29$, $df = 1$, $p = 0.021$; Xanthomonadaceae: $\chi^2 = 0.210$, $df = 1$, $p = 0.647$). Similar to the results at phyla level, family level relative abundances were similar across communities when the carcass surface, maggot mass, and internal larval communities were all present simultaneously (1557-1845 ADH) (Figs. 10 and 11). Additionally, the increase in relative abundance of *P. regina* larvae corresponded to the increase of Proteobacteria, specifically Xanthomonadaceae, in all of the microbial communities (Figs. 9, 10, and 11).

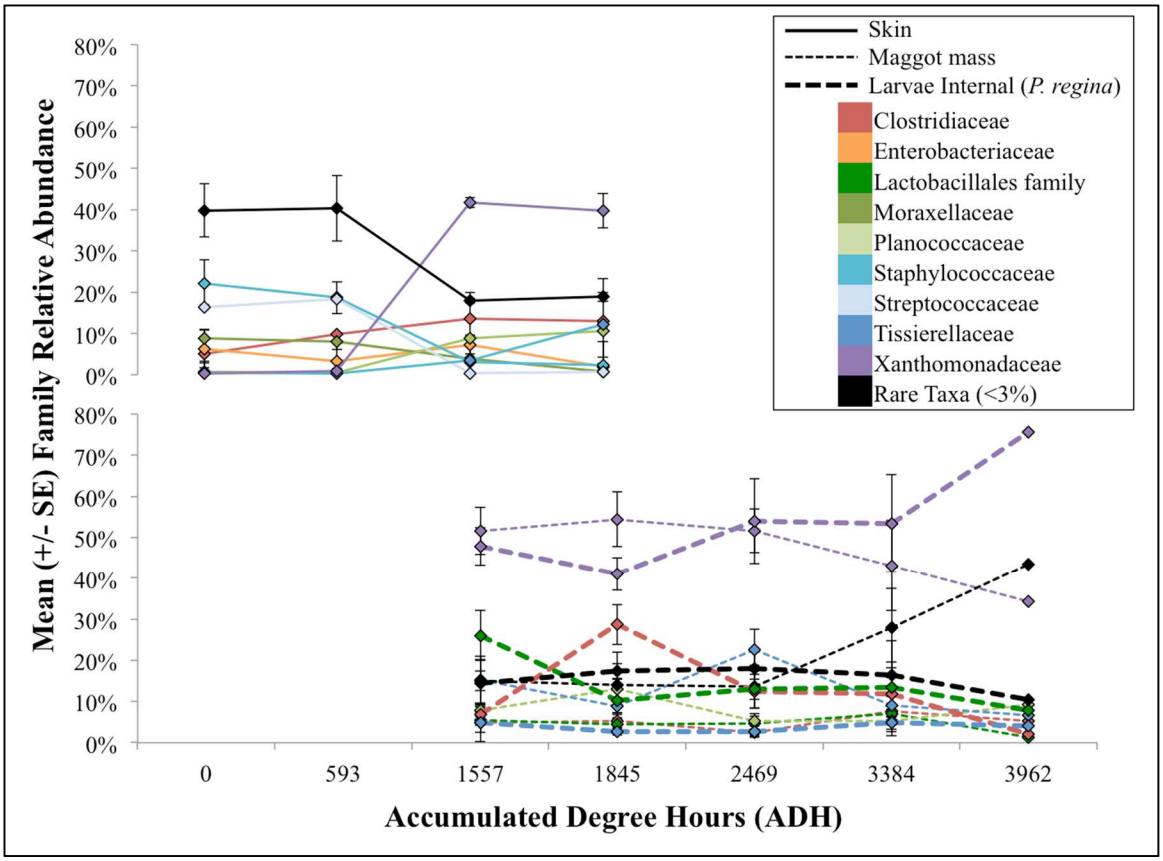

Figure 11: Mean (SE) relative abundance of dominant microbial families for carcass skin, maggot mass, and larval microbial communities over decomposition time expressed as accumulated degree hours (ADH). The top panel represents the skin microbial communities that were only present until 1845 ADH. The bottom panel shows the maggot mass microbial communities as well as those for *Phormia regina* larvae after the masses had formed.

Discussion

Past research has shown insects will colonize decomposing remains in a predictable succession (Payne 1965, Byrd and Castner 2001, Kreitlow 2009, Benbow et al. 2013), but there are few published findings of maggot mass species compositional changes throughout decomposition. Gruner et al. (2007) reported the co-occurrence of *L. coeruleiviridis*, *Chrysomya rufifacies* (Macquart) (Diptera: Calliphoridae), *Chrysomya megacephala* (Fabricius) (Diptera: Calliphoridae), *P. regina*, and *C. macellaria* larvae within masses located on swine carcasses during the summer in Florida. The authors found that *L. coeruleiviridis* was initially the most abundant blow fly species but decreased over time while *P. regina* increased throughout decomposition. However, they did not characterize the microbial communities associated with the carcass, larval mass, or individual larvae. Numerous studies have now shown the epinecrotic postmortem microbiome changes over time for a variety of animals including swine (*Sus scrofa* L.) (Benbow et al. 2015a, Pechal et al. 2013, 2014), mice (*Mus musculus* L., strain B6C3F1) (Metcalf et al. 2013, 2016), salmon (*Oncorhynchus keta* Walbaum) (Pechal and Benbow 2016), and humans (*Homo sapiens* L.) (Metcalf et al. 2016, Hyde et al. 2014).

Additionally, recent research has explored how insects are attracted to decomposing resources based on the volatile organic compounds (VOCs) produced by epinecrotic microbial communities (Zheng et al. 2013, Ma et al. 2012, Jordan et al. 2015). It is possible different necrophagous dipteran species prefer specific microbial communities and their emitted VOCs for their offspring and therefore arrive at different times following death (Jordan et al. 2015). This is consistent with Rosati (2014) who demonstrated that *P. regina* preferred to oviposit on a resource that had already been colonized by other calliphorid species. Similarly, in our study

there was a large increase in *P. regina* larval abundance later in decomposition, after other species had previously colonized. The successional shifts in relative abundance of each blow fly species over time might be explained by concurrent shifts in the microbial community of the carcass surface (epinecrotic microbial communities); however, this is an area where additional research into interdomain interactions associated with microbial and insect community succession on carrion is needed.

One of the most notable findings related to the microbial communities was the inverse relationship between the relative abundance of Firmicutes and Proteobacteria over the course of decomposition. This relationship was not unexpected as previous studies have documented similar results from terrestrial and aquatic swine carrion decomposition (Pechal et al. 2014, Benbow et al. 2015a), to the human gut (Del Chierico et al. 2015), and even inside the Ocean Cubiculum of the Roman Catacombs where the effect of blue light exposure on microbial communities was monitored over years (Urzi et al. 2014). The reason behind this relationship, however, is not yet fully understood and would be a fruitful area of future study.

There were also differential trends in taxa relative abundance among microbial communities on the carcass – skin, maggot mass, larval internal. When the maggot masses first formed, between 1557-1845 ADH, the internal and external larval microbiomes were similar to the skin microbial community composition. This suggests the larval internal microbes may have been acquired from their habitat and food source (i.e., the carcass), and that microbial communities can be trophically transferred during the decomposition process. It has already been shown that this microbial transfer is the case between the human diet and gut microbiome (Turnbaugh et al. 2009), and calliphorid and aquatic insect larvae in an aquatic habitat associated with high densities of decomposing salmon carcasses (Pechal and Benbow 2016).

Our results also suggest the carcass microbiome is influenced by insect colonizers. The sudden decrease of Firmicutes and increase of Proteobacteria relative abundances on the carcass surface did not occur until the first sampling point when maggot masses were initially present (1557 ADH). It has been well established that filth flies, such as Calliphoridae, can carry microbes and most notably transfer bacteria and disease causing pathogens to humans or food (Doud et al. 2014, Lilia et al. 2008, Graczyk et al. 2001, Blaak et al. 2014). If blow flies are bringing their own community of microbes to a carcass, it is probable that they are also taking microbes from the carcass and further dispersing them into the environment. Again, this is another area of interesting future research important to understanding carrion ecology with applications in disease ecology.

There is still a great need for further study in the area of microbe and insect community ecology of carrion, but our findings provide an important foundation to understanding the function of postmortem microbial communities and how they interact with the associated insect colonizers. A single larval mass is often comprised of a single family (e.g., Calliphoridae), but can contain multiples species with abundances that change throughout decomposition. Our finding that multiple necrophagous blow fly species co-occur as larvae on decomposing vertebrates has relevance in forensic entomology, where often only a few larvae are collected as evidence during death investigations; thus, there is the potential to miss additional species with different developmental characteristics that could affect period of insect activity estimates (Byrd and Castner 2001, Benbow and Tomberlin 2015). The finding that Xanthomonadaceae relative abundance increases throughout decomposition also has the potential to aid in estimating periods of insect activity and microbial succession. Typically Xanthomonadaceae are considered environmental organisms, including many plant pathogens and a few human pathogens (Pieretti

et al. 2009, LaSala et al. 2007). However, our findings, as well as those of similar studies, suggest that this family also plays an important role in decomposition (Metcalf et al. 2013, Pechal and Benbow 2016, Guo et al. 2016). Additionally, the microbiome of the carcass surface, the larval mass microbiome, and internal larval microbiome change temporally, potentially driving the changes in blow fly species abundance and transferring from one community to another becoming integrated into an ecosystem.

CHAPTER 3: ESTIMATING THE POST-COLONIZATION INTERVAL USING MULTI-SPECES CALLIPHORID LARVAL MASSES AND SPATIALLY DISTINCT TEMPERATURE DATA SETS

Abstract

Common forensic entomology practice is to collect the largest larvae from a scene and use temperature data from the nearest weather station with previously published larval development data in order to estimate development time. Insect growth is highly dependent on temperature, and yet, variation between temperatures sources and its effect on post-colonization interval (PCI) estimates is largely unknown. Additionally, other dipteran species are also present within larval masses but could be ignored by only collecting the largest specimens. In order to evaluate accuracy of PCI estimates among different Calliphoridae species and temperature sources, we sampled the larval communities of replicate swine carcasses (N = 6) every 12 h throughout decomposition and recorded ambient air temperature every 15 min at each carcass. Accumulated degree hour (ADH) range estimates were calculated using *Cochliomyia macellaria* and *Phormia regina* third instar presence and length with previously published developmental data. Actual ADH ranges were calculated using temperatures recorded from multiple sources at varying distances from the study site: individual temperature loggers at each carcass, a local weather station, and a regional weather station. The PCI estimate successfully encompassed the average ADH for each temperature source and species but overall underestimated each actual ADH range. Little variation existed between *C. macellaria* and *P. regina* accuracy. Third instars varied greatly in size and abundance but for both species, the ADH estimate derived from the 50th percentile length, was close to the actual minimum ADH for all temperatures sources. These

results provide an important step towards improving evidence collection and analysis techniques, and developing forensic error rates.

Introduction

Determining time of death is a significant element of solving crimes yet it is a difficult piece of information to estimate or predict using existing, and often fragmented, evidence collected during a death scene investigation. Patterns of postmortem insect succession and development are common tools used to narrow the estimated time of death, providing investigators with a time range commonly referred to as the post-mortem interval (PMI) (Byrd and Castner 2001, Catts and Haskell 1990). Indeed, insects provide invaluable clues towards narrowing time of death, however, the term PMI can be misleading as it implies the entire time since death (Tomberlin et al. 2011b). Insect evidence can only provide information on the amount of time insects have been present (i.e. how long the resource has been colonized) which is not necessarily the same as time since death (Amendt et al. 2007, Tomberlin et al. 2011b). Death scenes are highly variable and immediate insect colonization following death cannot always be assumed. For example, the arrival of insects can be delayed if death occurs indoors (Anderson 2011), if the deceased is wrapped (Goff 1992), or if the remains are altered by chemicals (Bugajski 2011), or burning (Avila and Goff 1998). Therefore, we use the term post-colonization interval (PCI) here as it refers solely to the time from insect colonization to discovery of the remains (Tomberlin et al. 2011b).

After colonization, the greatest influence on blow fly larval development is temperature since insects are exothermic (Byrd and Castner 2001). Therefore, the amount of time required for Calliphoridae larvae to develop depends on the temperature of their environment. In order to standardize development time, temperatures are commonly converted to accumulated degree hours (ADH) using a thermal summation model. However, there are many other factors, both biotic and abiotic, that can influence larval development making it difficult to work backwards in

order to estimate the initial time of colonization (Catts 1992). For example, rain (Mahat et al. 2009), drugs (Goff et al. 1989, 1991, 1992), predation (Greenberg 1991), larval sex (Picard et al. 2013), geographic location and thus genetics (Owings et al. 2014, Tarone and Foran 2006), tissue type (Clark et al. 2005), and bacteria (Ahmad et al. 2006) can all affect larval development.

With all this potential for variability, the Daubert standard (Daubert v. Merrell Dow Pharmaceuticals 1993) and the 2009 National Academy of Sciences Report (National Research Council 2009) suggest that, variability studies, such as this, are crucial to understanding how different factors affect insect development so that proper evidence collection and analysis techniques can be established in order to estimate the post-colonization interval as accurately as possible (Tomberlin et al. 2011a, 2011b). Currently, investigators at the scene will often collect just a few of the largest larval specimens from a single location and, therefore, could be missing essential information for developing a more concise PCI estimate (Byrd and Castner 2001, Benbow and Tomberlin 2015). Studies have shown that a larval mass can contain multiple blow fly (Diptera: Calliphoridae) species whose abundance changes over time (Gruner et al. 2007). By only collecting the largest specimens, entire species, smaller yet potentially more developed, could go unnoticed.

Another common practice in forensic entomology is to use the nearest weather station to the death scene and previously published larval development data in order to determine insect developmental time (Byrd and Castner 2001, Catts and Haskell 1990). While a weather station is the best source for historical temperature data, it cannot be assumed that the station accurately reflects the temperature of the scene (Baker et al. 1985, Catts 1992). Temperature variation between the site and weather station could potentially affect the accuracy of PCI estimates.

The objective of this study was to assess the accuracy of PCI estimates, derived from previous developmental data for multiple Calliphoridae species collected from a single larval mass, when utilizing different data sets (i.e. individual carcass, microhabitat, local, regional temperature) at varying distances from a carcass. We predicted that different Calliphoridae species would colonize the resource at different times and that the temperature source closest to the carcasses would record temperatures most similar to what the larvae experience. Therefore, we hypothesized that different species would yield different ADH ranges and that the closest temperature data set to the carcasses would result in a more accurate PCI estimate.

Materials and Methods

Study Site

The study was conducted from 19-27 August 2014 at Purdue University's Forensic Entomology Research Compound in West Lafayette, Indiana (40°25'36.0"N, 86°56'57.0"W). Six (three male and three female) swine carcasses (*Sus scrofa* L.), averaging 19.65 kg (± 5.44 SD) and approximately 80.0 cm in length, were purchased from a local farm and therefore exempt from university IACUC review. Carcasses were immediately placed in plastic bags during transport to the site (approximately 4 h), as previously described (Benbow et al. 2013, Pechal et al. 2013, Pechal et al. 2014a, Pechal et al. 2014b). The carcasses were placed on two East-West running transects approximately 50 m apart in a grassy field surrounded by deciduous forest. All carcasses were oriented on their left side with heads directed north and placed on top of stainless steel grates (~1.0 m^2), with ~5.0 cm^2 openings, located on the ground to allow for lifting the individual carcasses during sample collection. Cages (~1.0 m^3) constructed of chicken mesh wire and treated lumber were secured over the carcasses and the grates to prevent vertebrate scavenging. Insect communities were sampled (see below) every 12 h at approximately 0700 h and 1900 h for 8 consecutive days, which was the amount of time for the carcasses to fully decompose to only bones and skin, and dipteran larvae to develop and begin the post-feeding dispersal from the resources for pupation.

Temperature

The ambient temperature at each carcass was recorded every 15 min using one HOBO™ temperature logger (Onset Computer Corporation, Bourne, MA, USA) attached to the east side

of each anti-vertebrate scavenging cage approximately 0.90 m off the ground. Temperatures from all 6 loggers were averaged to determine the temperature of the microhabitat. Local temperature data were collected from the Purdue University Airport (40°24’48.5”N, 86°56’26.1”W) weather station, located 1.63 km southeast of the study site. Regional temperature data were collected from a Lafayette, Indiana weather station (MAU199) located 7.61 km southeast of the study site (40°22'40"N, 86°53'13"W) (Fig. 12). Both local and regional temperatures were recorded at least once every hour throughout the study.

Larvae

At each sampling period, blow fly larvae were collected using a single cotton-tipped swab (Puritan Medical Products, Guilford, ME, USA) from three different areas of a single larval mass located on the carcass, and stored at -20 °C. Third instars were identified to species level (Stojanovich et al. 1962, Wells et al. 1999) and then stored in 100% molecular grade ethanol. *Cochliomyia macellaria* (Fabricius) (Diptera: Calliphoridae) and *Phormia regina* (Meigen) (Diptera: Calliphoridae) were used for PCI analysis due to specimen quantity and previously published development data availability (Byrd and Butler 1996, Byrd and Allen 2001). Each larva was photographed on a 2 mm micrometer slide. Larval body lengths were then measured to the nearest 0.001 mm in ImageJ 1.46r (Schneider et al. 2012) by setting the scale using the micrometer and measuring a line drawn through the larval midline (Fig. 13). It is important to note that multiple people determined and measured midlines and thus, it is a potential source of variability and error.

Figure 12: Map showing the location of the microhabitat (HOBO temperature loggers), local (Purdue University Airport weather station), and regional (Lafayette weather station) temperature sources.

Figure 13: Example of third instars photographed on a micrometer slide and measured in ImageJ. Black lines indicate the midline that was measured in order to determine length.

Post-Colonization Intervals

All PCIs represent either the actual or estimated ADH range of third instars since that is the stage most commonly collected by investigators and used for identification. Estimated ranges refer the expected ADH required for a specific species to reach a certain development stage based off of previous data sets. Actual ranges refer to the actual ADH that third instars were present during this study. Estimated third instar ADH ranges for *C. macellaria* and *P. regina* were determined using previously published hourly ranges for the third instar (Byrd and Butler 1996, Byrd and Allen 2001). Developmental data sets were chosen based on the range of developmental information provided and proximity to study site. For instance, hourly ranges for larvae reared at 25 °C were used for both species as the overall average temperature recorded at the carcasses throughout decomposition was 25.69 °C (± 0.257 SE) (Fig. 14). Actual ADH were calculated by adding the hourly temperatures minus the minimum threshold for each species, starting at time of carcass placement. A minimum threshold of 10 °C was used for *C. macellaria* (Greenberg 1991, Boatright and Tomberlin 2010) and 6 °C for *P. regina* (VanLaerhoven 2008). Ranges were determined based off of the ADH that had been acquired by the first and last sampling points when third instars were collected for each carcass. In order to assess variability, ranges were calculated for each temperature source: the individual carcass, the microhabitat, local, and regional temperature data. Time point 0 represented when the carcasses were placed in the field and insects were allowed access at approximately 4 h postmortem.

Third instar length was also utilized to form PCI estimates as this is an additional measurement used in forensic investigations. As previously published length developmental data is only available in line graph form (Byrd and Butler 1996, Byrd and Allen 2001), length to time conversion relies on visual assessment and is therefore subjective. In order to reduce bias, we

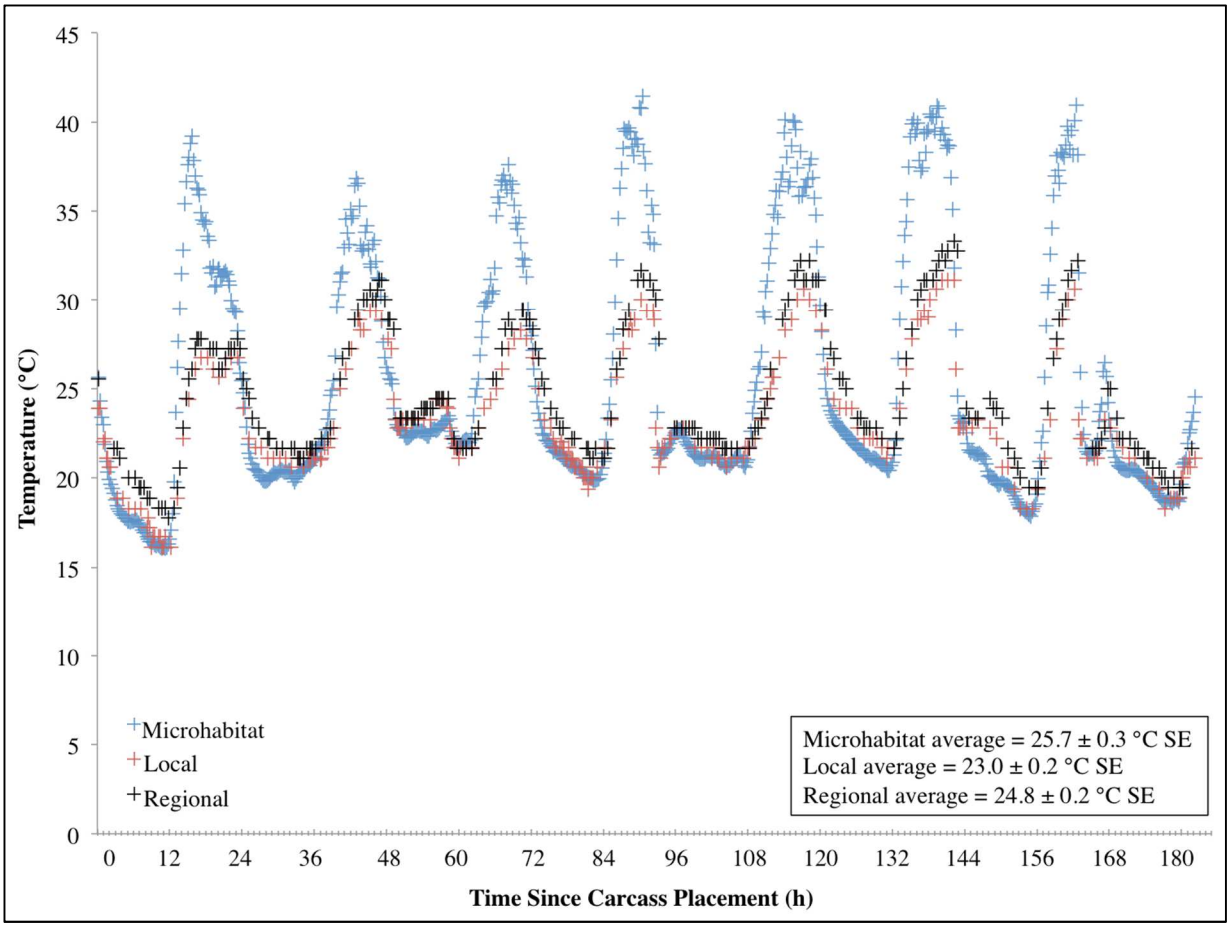

Figure 14: Temperature (°C) throughout decomposition for the microhabitat, local, and regional sources. Microhabitat was recorded every 15 min while local and regional were recorded at least hourly.

formed estimates using the average 10^{th}, 50^{th}, and 90^{th} percentile lengths of third instars collected from all carcasses for each calliphorid species. From these lengths, the associated ADH were determined by visual assessment of previously published 25 °C growth curves (Byrd and Butler 1996, Byrd and Allen 2001) and minimum thresholds of 10 °C (Greenberg 1991, Boatright and Tomberlin 2010) and 6 °C for *C. macellaria* and *P. regina* (VanLaerhoven 2008), respectively.

Statistical Analysis

Repeated measures analysis of variance (ANOVA) was conducted using SAS Studio 3.5 (SAS Institute Inc., Cary, NC, USA) to compare differences in temperature among the 3 locational data sets (micro, local, regional) and among the individual carcasses. A t-test was performed in SAS to compare differences in temperature among the local and regional sources over time. Correlations in the relative abundance of *C. macellaria* and *P. regina* third instars were evaluated using a Spearman Rank test. Coefficient of variation (CV) was calculated to assess temperature variability at each source and third instar length variability at each time point for each species. All p-values were considered significant with alpha at or below 0.05.

Results

Temperature

The average temperatures of the micro, local, and regional habitats throughout the study were 25.7 °C (± 7.01 SD), 23.5 °C (± 3.50 SD), and 24.6 °C (± 3.74 SD), respectively. Microhabitat temperatures were more variable (0.27 CV) than both local (0.15 CV) and regional (0.15 CV) weather station temperatures. Throughout the duration of decomposition, there was little visual variation between temperatures recorded at the local and regional sources, however, temperature differences were significant ($t = -12.19$, $p = <0.0001$). All three sources recorded similar daily minimum temperatures (microhabitat: 19.5 ± 1.89 °C SD, local: 19.9 ± 2.06 °C SD, regional: 20.7 ± 1.40 °C SD). However, the microhabitat consistently recorded higher daily maximum temperatures (39.1 ± 1.65 °C SD) than local (29.5 ± 1.56 °C SD) and regional (31.1 ± 1.89 °C SD) (Fig. 14). There were significant differences among temperatures recorded at the 6 carcasses ($F = 38.31$, $df = 187$, $p = <0.0001$) and among the micro, regional, and local temperature sources. ($F = 10.22$, $df = 184$, $p = <0.0001$).

Calliphoridae Larvae

Calliphoridae egg clutches were first observed on carcasses 2 and 3 at 20 - 30 min after placement and on all other carcasses by 12 h later. Third instars were first collected as early as 48 h after carcass placement and as late as 156 h. A total of 904 third instars were identified: 258 *C. macellaria,* 509 *P. regina,* and 137 *Lucilia coeruleiviridis* (Macquart) (Diptera: Calliphoridae). All third instar *C. macellaria* and *P. regina* were measured and used for PCI calculations. The greatest number of *C. macellaria* third instars were present at 48 – 60 h following carcass placement. Abundance of *P. regina* increased shortly thereafter (72 h) and then

decreased (84 – 108 h) before increasing again (120 h). *C. macellaria* third instar average relative abundance was greatest at 48 h (59.51%) while *P. regina* relative abundance remained above 60.00% for a majority of the third instar hourly range (84 -156 h) (Fig. 15). There was a significant corrleation between *C. macellaria* and *P. regina* third instar average relative abundance throughout decomposition ($\rho = -0.9$, $p = 0.0002$). The average relative abundance of third instars, compared to first and second instars, increased over time with the exception of a decrease at 96 h after carcass placement (Fig. 16).

There was great variability among third instar lengths. The overall range of lengths for *C. macellaria* was 4.28 – 14.53 mm and 3.11 – 12.22 mm for *P. regina*. Even within a single sampling point, there were large differences in lengths for each species. The greatest differences observed within a single sampling point were 4.80 – 14.53 mm at 60 h and 3.62 – 11.55 mm at 72 h following carcass placement for *C. macellaria* and *P. regina,* respectively. The average length for each species, however, changed little over time (*C. macellaria*: 7.63 ± 0.63 mm SD, *P. regina*: 7.33 ± 0.68 mm SD). The variability in third instar lengths at each sampling point (48, 60, 72, 84, 96, 108, 120, 132, 144, 156 h following carcass placement) fluctuated over time for *C. macellaria* (CV = 0.079, 0.212, 0.219, 0.233, 0.203, 0.147, 0.000, 0.256, 0.105, 0.193) but the variability in *P. regina* lengths remained fairly consistent throughout decomposition (CV = 0.083, 0.223, 0.225, 0.212, 0.245, 0.243, 0.266, 0.281, 0.277, 0.197) (Fig. 17).

Post-Colonization Intervals

The PCI estimates derived from Byrd's data sets most often fell within the ranges derived from local temperatures for *C. macellaria* and regional temperatures for *P. regina*. For both

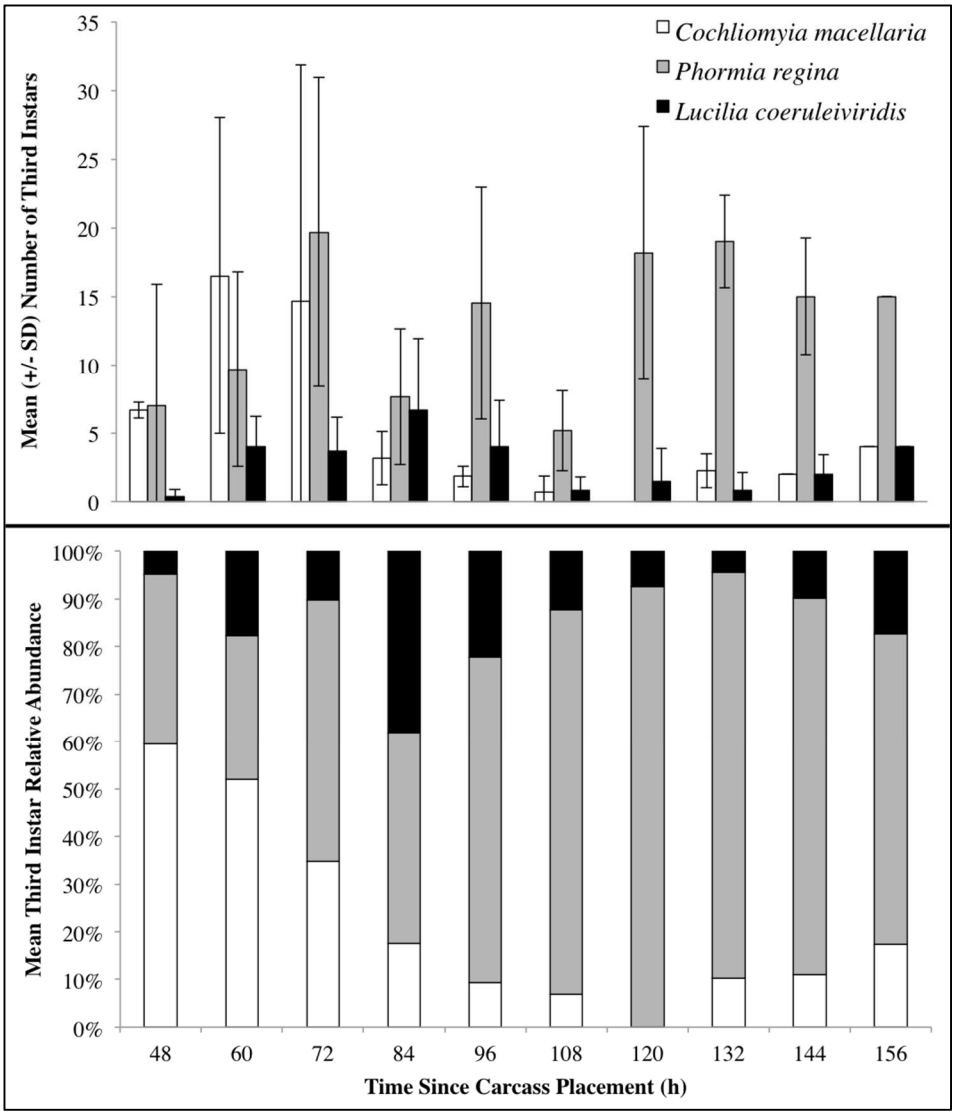

Figure 15: Mean (SD) number (top panel) and relative abundance (bottom panel) of all third instars collected throughout decomposition: *Cochliomyia macellaria, Phormia regina,* and *Lucilia coeruleiviridis.*

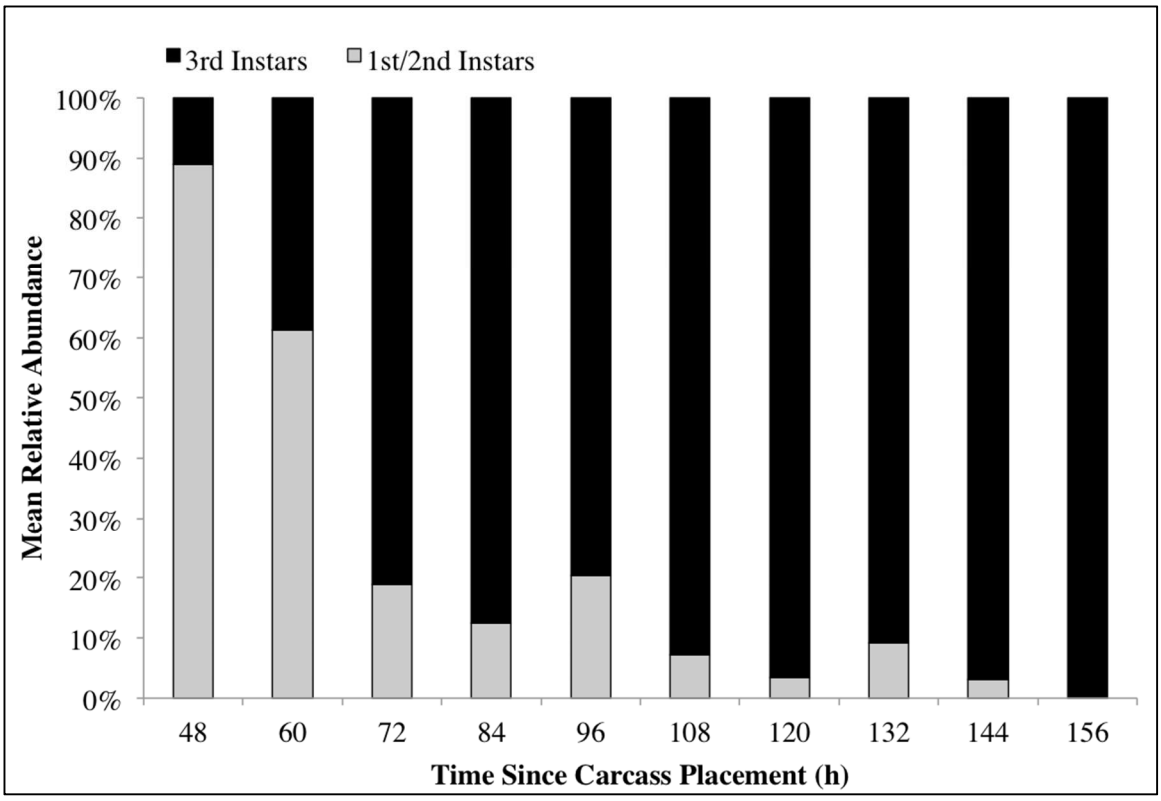

Figure 16: Relative abundance of first/second and third instars collected from all carcasses throughout decomposition.

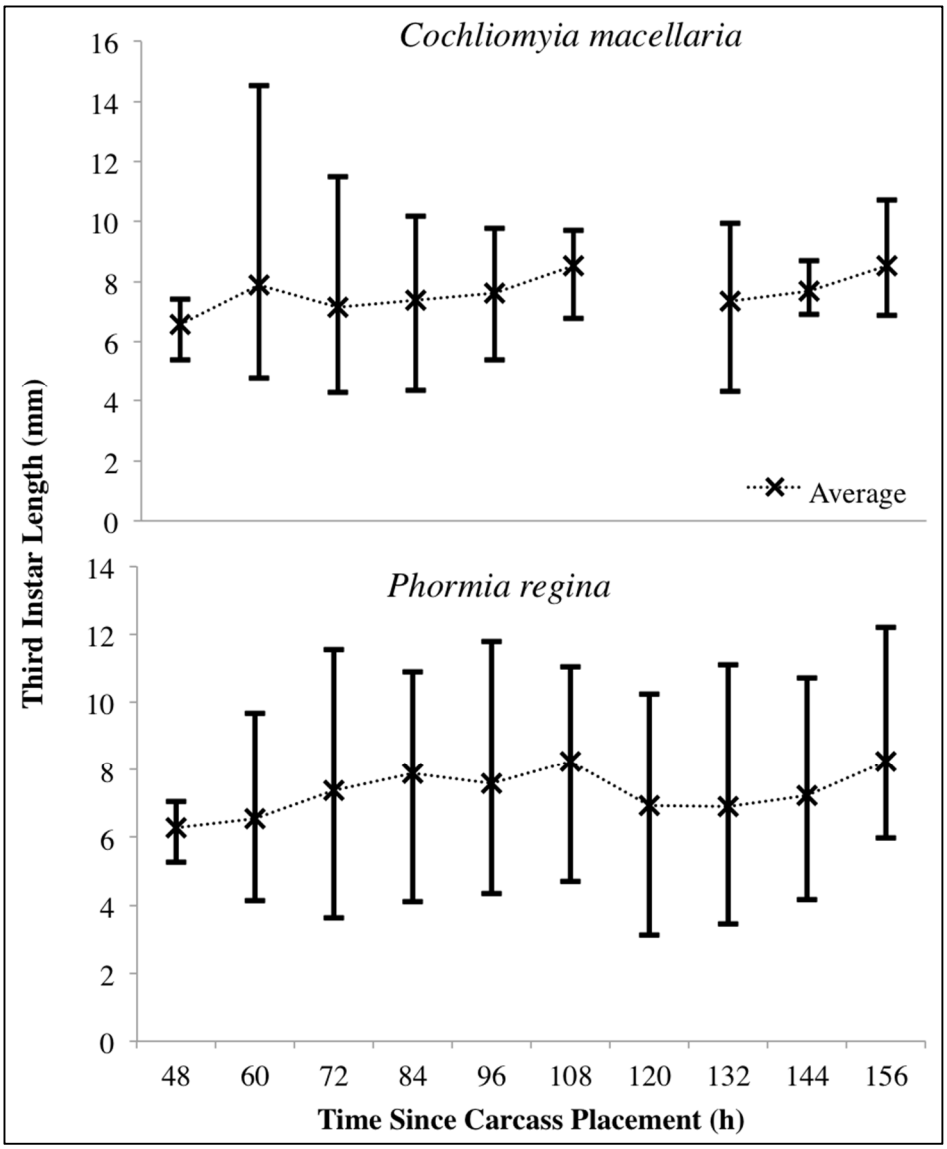

Figure 17: Range of *Cochliomyia macellaria* and *Phormia regina* third instar lengths throughout decomposition. Vertical lines represent the range of larvae collected at that time point for all carcasses. Upper and lower limits represent the maximum and minimum lengths, respectively. Average lengths are marked with an "X".

species, microhabitat temperatures produced the widest ADH range. The PCI estimates for both species underestimated the minimum ADH for all temperature sources. The maximum ADH was also underestimated when microhabitat and regional temperatures were used with *C. macellaria* and with microhabitat temperatures for *P. regina* (Figs. 18 and 19). For *C. macellaria,* the PCI estimate derived from Byrd and Butler (1996) accounted for 75.7%, 88.5% and 82.8% of the actual ADH range for microhabitat, local, and regional temperatures, respectively. For *P. regina,* the PCI estimate derived from Byrd and Allen (2001) accounted for 86.8%, 88.9% and 93.4% of the actual ADH range for microhabitat, local, and regional temperatures, respectively. Overall, the PCI estimates underestimated the actual ADH but did encompass the average ADH for all temperature sources (Tables 5 and 6).

For estimates derived using third instar length and previous developmental data (Byrd and Butler 1996, Byrd and Allen 2001), larval lengths at the 10th percentile (*C. macellaria:* 5.36 ± 0.36 mm SD, *P. regina:* 5.44 ± 0.92 mm SD) for both species underestimated the minimum ADH for all temperatures sources. The 50th percentile lengths (*C. macellaria:* 7.21 ± 0.74 mm SD, *P. regina:* 6.95 ± 1.18 mm SD) of both species fell within local and regional temperature ADH ranges and just under the microhabitat minimum ADH. The 90th percentile length (*C. macellaria:* 9.66 ± 0.42 mm SD, *P. regina:* 9.33 ± 0.90 mm SD) for both species fell within the range from all sources. For both species, the ADH derived from the 50th percentile length was close to the actual microhabitat and regional minimum ADH. None of the percentile lengths were near any of the maximum ADH range (Figs. 18 and 19).

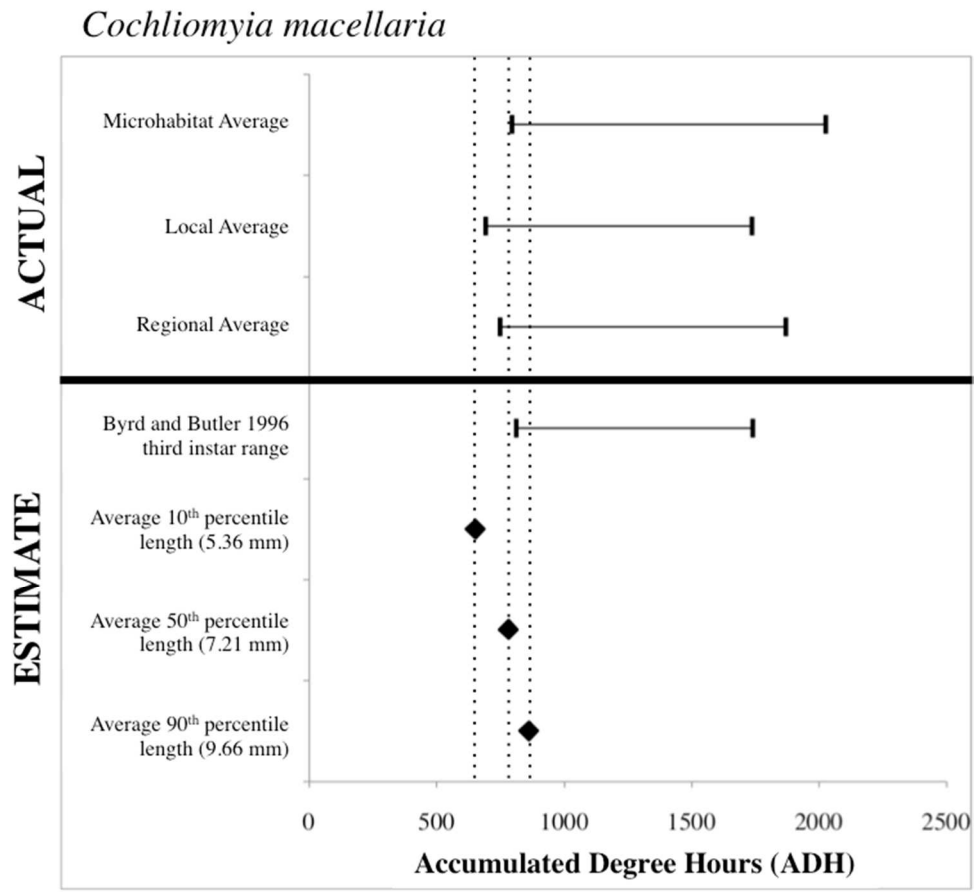

Figure 18: Comparison of post-colonization interval (PCI) estimates, determined using *Cochliomyia macellaria* third instar developmental stage and length, to actual PCIs calculated for each temperature source: microhabitat, local, and regional.

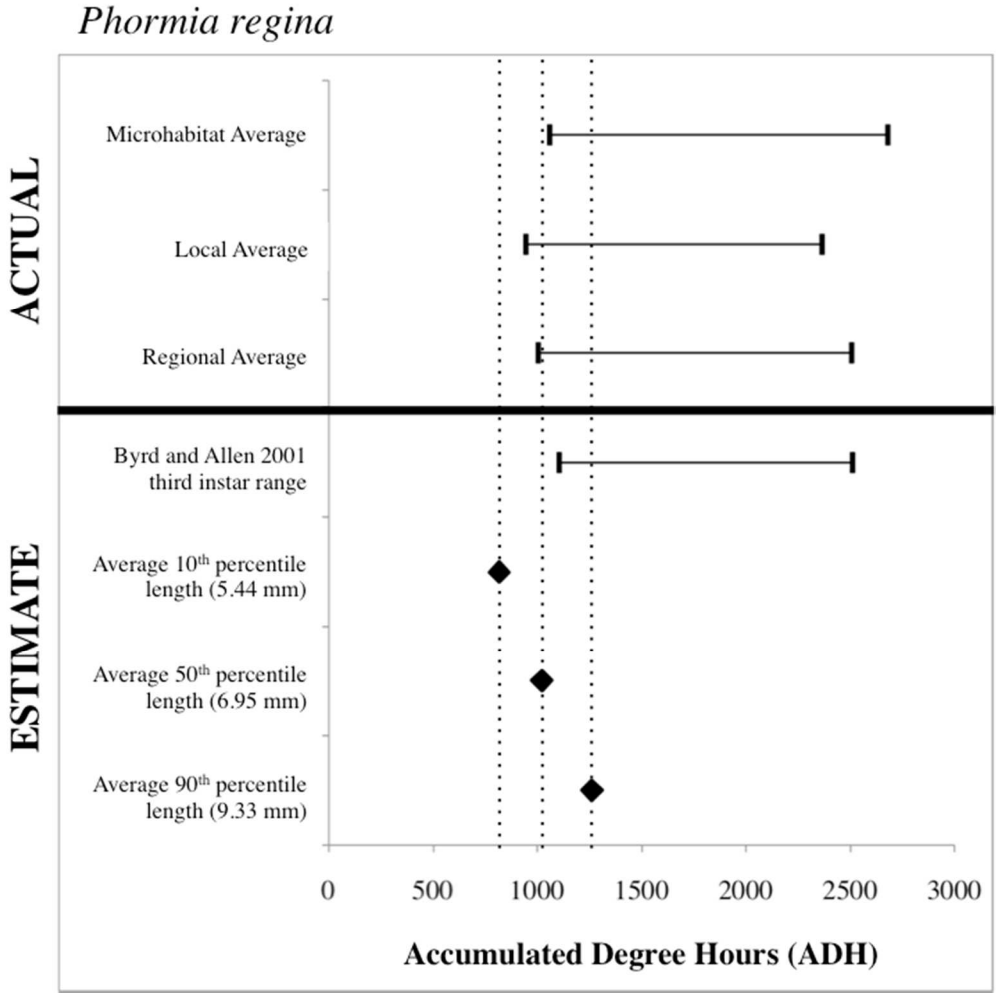

Figure 19: Comparison of post-colonization interval (PCI) estimates, determined using *Phormia regina* third instar developmental stage and length, to actual PCIs calculated for each temperature source: microhabitat, local, and regional.

Table 5: Actual and estimated accumulated degree hours (ADH) for *Cochliomyia macellaria* based on third instar range and lengths. White areas represent actual values while gray areas represent estimated values (Byrd and Butler 1996).

Carcass	3rd Instar Range (h)	Individual Range (ADH)	Individual Median (ADH)	Micro Range (ADH)	Micro Median (ADH)	Local Range (ADH)	Local Median (ADH)	Regional Range (ADH)	Regional Median (ADH)	Byrd 3rd Instar Range at 25°C (h)	Byrd Range (ADH)	Byrd Median (ADH)	10th percentile length (mm)	10th percentile ADH	50th percentile length (mm)	50th percentile ADH	90th percentile length (mm)	90th percentile ADH
1	48-156	727-2423	1575	718-2487	1603	610-2132	1371	663-2300	1482	54-116	810-1740	1275	5.698	690	7.398	780	10.084	870
2	48-96	706-1510	1108	718-1506	1112	610-1282	946	663-1386	1025	54-116	810-1740	1275	5.832	690	7.110	780	9.225	840
3	48-108	743-1750	1247	718-1641	1180	610-1422	1016	663-1532	1098	54-116	810-1740	1275	5.306	630	7.164	780	9.557	855
4	60-132	916-2129	1523	871-2071	1471	770-1787	1279	830-1922	1376	54-116	810-1740	1275	5.365	630	8.343	810	10.222	885
5	60-144	846-2274	1560	871-2370	1621	770-2003	1387	830-2157	1494	54-116	810-1740	1275	5.020	630	7.206	780	9.657	870
6	60-132	831-1990	1411	871-2071	1471	770-1787	1279	830-1922	1376	54-116	810-1740	1275	4.939	630	6.026	750	9.237	840
Average	56-134	795-2013	1404	795-2024	1410	690-1736	1213	747-1870	1309	54-116	810-1740	1275	5.360	650	7.208	780	9.664	860
Coefficient of Variation			0.135		0.152		0.154		0.152				0.066	0.048	0.103	0.024	0.043	0.021

Table 6: Actual and estimated accumulated degree hours (ADH) for *Phormia regina* based on third instar range and lengths. White areas represent actual values while gray areas represent estimated values (Byrd and Allen 2001).

Carcass	3rd Instar Range (h)	Individual Range (ADH)	Individual Median (ADH)	Micro Range (ADH)	Micro Median (ADH)	Local Range (ADH)	Local Median (ADH)	Regional Range (ADH)	Regional Median (ADH)	Byrd 3rd Instar Range at 25°C (h)	Byrd Range (ADH)	Byrd Median (ADH)	10th percentile length (mm)	10th percentile ADH	50th percentile length (mm)	50th percentile ADH	90th percentile length (mm)	90th percentile ADH
1	48-156	898-3047	1973	910-3111	2011	802-2756	1779	855-2924	1890	58-132	1102-2508	1805	5.999	855	8.323	1216	10.338	1311
2	48-120	919-2395	1657	910-2408	1659	802-2109	1456	855-2233	1544	58-132	1102-2508	1805	5.285	836	6.600	874	9.606	1273
3	48-120	935-2563	1749	910-2408	1659	802-2109	1456	855-2233	1544	58-132	1102-2508	1805	4.937	836	6.768	1007	9.027	1254
4	60-132	1156-2657	1907	1111-2599	1855	1010-2315	1663	1070-2450	1760	58-132	1102-2508	1805	5.974	855	8.046	1197	9.974	1292
5	60-144	1086-2850	1968	1111-2946	2029	1010-2579	1795	1070-2733	1902	58-132	1102-2508	1805	6.500	874	6.903	988	9.283	1254
6	72-132	1365-2518	1942	1406-2599	2003	1241-2315	1778	1311-2450	1881	58-132	1102-2508	1805	3.933	646	5.036	836	7.760	1178
Average	56-134	1060-2672	1866	1060-2679	1869	945-2364	1655	1003-2504	1754	58-132	1102-2508	1805	5.438	817	6.946	1020	9.331	1260
Coefficient of Variation			0.071		0.093		0.097		0.097				0.170	0.104	0.169	0.156	0.097	0.037

Discussion

The cornerstone of forensic entomology and determining a post-colonization interval is that necrophagous fly species develop at specific and predictable rates under certain temperatures (Kamal 1968, Anderson 2000). However, temperature history of a crime scene is not always available. Instead, investigators use temperature data collected from the nearest weather station, potentially kilometers away, to calculate ADH that are then compared to developmental data sets generated in a controlled laboratory setting (Byrd and Castner 2001, Catts and Haskell 1990). Recently the forensic science community has increased efforts to recognize the amount of variability in specific disciplines and develop error rates (National Research Council 2009). Yet, it is still relatively unknown how the accuracy of developmental data set PCI estimates varies depending on temperature source. Archer (2004) examined the accuracy of collecting scene temperatures after body removal and retrospectively correcting weather station data. Often minimum PMI estimates improved following correction but temperature collection after body discovery is not always possible. Archer urges caution when correcting since weather can change significantly over a short period of time. Improvement of estimates was highly variable among correlation periods and in a few instances, temperature correction was associated with a decrease in estimate accuracy. Additionally, only hypothetical insect data were analyzed (Archer 2004).

Contrary to our hypothesis, using temperature sources closer to the carcasses did not result in an increase in PCI estimate accuracy. PCI estimates determined using previous developmental data sets were more similar to the actual third instar range when weather station temperatures were used rather than temperatures from the scene. The estimates underestimated the total microhabitat ADH range, which was overall larger than the weather station ranges. This wider ADH range at the microhabitat level could be a result of the fact that the temperature

loggers were closer to the ground and more often shaded than the weather stations, resulting in higher temperatures and thus more rapid accumulation of degree hours (Byrd and Castner 2001). The greatest difference in temperatures between the sources occurred during the afternoon, when temperatures were highest. While the accuracy of the PCI estimates varied greatly in relation to the maximum ADH, the minimum ADH was similar across all temperatures sources. This is beneficial for forensics as the minimum amount of time is what is commonly determined by forensic entomologists (Byrd and Castner 2001).

Regardless of the temperature source used to calculate ADH ranges for use in PCI estimates, there is already probable inaccuracy due to the fact that the developmental data sets used to calculate the PCI estimates (Byrd and Butler 1996, Byrd and Allen 2001) were generated in a controlled laboratory setting with constant temperatures (Byrd and Castner 2001). This is in contrast to the field conditions where temperatures fluctuate and development can be influenced by more variables. Our findings suggest that trying to fit a loose fitting ADH range to more precise temperature data results in a more inaccurate estimate. There were fewer fluctuations in temperature from the weather stations than at the microhabitat, making it more similar to laboratory conditions where there were no fluctuations. These two sources of error may be canceling each other out as frequently errors will occur in both directions, one may overestimate while the other underestimates development time (Higley et al. 1986).

Another notable finding was the variation in species and third instar length. This supports the importance of thorough evidence collection in order to utilize as much information available as possible. The largest larvae may be the most developed and therefore an initial colonizer, but the presence of smaller third instars, of either the same or different species, could indicate multiple oviposition events that could help develop a more complete timeline. Third

instars were present for a wide range of time (hours) yet the relative abundances of thirds compared to other instars shifted throughout decomposition. There was an increase in relative abundance of first and second instars at 96 h and then an increase in abundance of third instar *P. regina* at 120 h, suggesting a second oviposition event occurred (Richards and Villet 2008). Smaller third instars of another species could be informative as different species colonize at different times following death. *P. regina* is known to be a later colonizer, compared to *L. coeruleiviridis, C. macellaria,* and *Chrysomya rufifacies* (Macquart) (Diptera: Calliphoridae) (Gruner et al. 2007), and prefers a resource that has been previously colonized (Rosati 2014). We initially observed a high average relative abundance of *C. macellaria* (59.51%) early on (48 h) followed by an increase in *P. regina* average relative abundance (55.01% at 72 h). Also, the range of lengths was variable but the average length showed little fluctuation throughout decomposition, supporting the idea that a more thorough sampling, rather than just the largest larvae, would result in a more accurate analysis of development (Byrd and Castner 2001).

Using the 50^{th} and 90^{th} percentile lengths of third instars was effective at indicating the lower ADH range but failed to express the upper ADH range. A possible explanation could be preservation methods. Specimens were frozen and then placed in ethanol, which could have caused them to shrink and produce underestimated ranges (Tantawi and Greenberg 1993). It would be useful to replicate this study and compare ADH estimates derived from length among different preservation methods. This knowledge would be valuable as experience and technique varies among investigators. It is not uncommon for an entomologist to receive poorly preserved specimens.

This study is insightful in that it demonstrates the vast variability present in Calliphoridae larval development and temperature among locations, but it also raises a number of questions.

For example, weather station temperature data produced better PCIs but it is not yet fully understood why microhabitat temperatures were less accurate or how widespread this phenomenon is. Continued research and replication would be beneficial towards improving collection and analysis of entomological evidence.

CHAPTER 4: CONCLUSION

In summary, this research has shown that carrion provides a rich environment for both interdomain and interspecies interactions. This study has demonstrated a high degree of temporal change and variability with respect to many elements within carrion ecosystems: microbial and insect community composition, insect development, and temperature. Multiple Calliphoridae species utilize this ephemeral resource simultaneously and interact at varying abundances throughout decomposition. Microbial communities change over time, likely, in part to their interactions with the insect communities. Temperatures can fluctuate over just a short distance and there is great variation within a single developmental stage of a single species. PCI estimates determined using previous developmental data sets were more similar to the actual third instar range when weather station temperatures were used rather than temperatures from the scene. All of this information helps provide a fundamental basis towards a greater understanding of the effects of carrion in an ecosystem and how this information can be applied to forensic investigations. We know large variability can exist throughout the decomposition process and insect development but it is still not completely understood how it ultimately affects ecosystems and forensic estimates. There is still a lot of research to be conducted and a lot to be discovered.

There are many directions that could be pursued if this research continued. First, the microbiome of adult blow flies, both those arriving and those emerging from the carcass, could be examined. We suspect the postmortem epinecrotic microbiome is affected by the presence of larvae. It would be useful to know if the arriving adult flies carry microbes similar to what is found on the carcass surface. It is also suspected that larvae acquire part of their microbiome from the carcass so it would be beneficial to know if the transfer of microbes continues to the

adult stage and what is being further dispersed into the ecosystem. Ultimately asking, where are the microbes coming from and where are they going?

It would also be important to replicate the study in a different geographical location, a different season, or a different landscape (e.g. forest, open field). In order for research to successfully be applied to a wide-range of forensic investigations, especially post-colonization estimates, it is necessary to understand if the observed phenomenon occurs every time or only in certain environments. In this study, microhabitat temperatures in the afternoons were warmer than the weather station locations but perhaps results would have been different if the field where carcasses were placed was not completely surrounded by trees.

Furthermore, more thorough sampling of maggot masses could add robustness to our species abundance or larval development findings. Previously, larval samples were collected from only the surface of the mass. If specimens were collected from within the center of the mass, would the same ratio of species be observed with the same developmental ranges? Information such as this could be critical for teaching investigators how to collect evidence.

Last but not least, delving deeper into the larval and microbial interactions occurring throughout decomposition would be fruitful. Trends were demonstrated with this research but the reasons behind them are still unconfirmed. The question remains, are these microbial communities dictating insect colonization or development? In order to utilize entomological findings for forensic investigations it is important to not only understand what you find but why. For example, knowing how to analyze a wide range of third instar lengths for PCI estimates is important but equally so is understanding why so much variation in development exists.

The potential for future carrion decomposition research is endless. Within this study alone, the possibilities to expand are vast. Despite all the current knowledge, I believe we are just beginning to understand this complex ecosystem.

APPENDIX

APPENDIX

RECORD OF DEPOSITION OF VOUCHER SPECIMENS

The specimens listed below have been deposited in the named museum as samples of those species or other taxa, which were used in this research. Voucher recognition labels bearing the voucher number have been attached or included in fluid preserved specimens.

Voucher Number: 2016-04

Author and Title of thesis:

Courtney R. Weatherbee

"Ecology of Calliphorid Larval Masses and Postmortem Colonization Estimate Variability"

Museum where deposited:

Albert J. Cook Arthropod Research Collection, Michigan State University (MSU)

Specimens:

Family	**Genus-Species**	**Life Stage**	**Quantity**	**Preservation**
Calliphoridae	*Cochliomyia macellaria*	Larva	10	Alcohol
Calliphoridae	*Lucilia coeruleiviridis*	Larva	10	Alcohol
Calliphoridae	*Phormia regina*	Larva	10	Alcohol

LITERATURE CITED

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