

## **ABSTRACT**

CRUISE, ANGELA MARIE. Sampling and ecological succession of adult necrophilous insects. (Under the direction of Dr. Coby Schal and Dr. Wes Watson).

Forensic entomology links the science of entomology and the judicial system, most notably in death investigations. Using entomological evidence from a crime scene and biotic and abiotic characteristics of the environment, forensic investigators make inferences about the time since death, or the postmortem interval (PMI). Understanding parameters such as geographic location and level of concealment enables the prediction of necrophilous insect succession on a decomposing body, which is essential to PMI determination.

Of particular need in forensic entomology are studies examining the pre-colonization interval and mechanisms driving insect ecological succession. The characterization of ecological succession in general, and of necrophilous insects, depends on accurate documentation of temporal and spatial changes in species diversity, richness, and abundance. Pigs are the most common models used in forensic studies because human cadavers and pigs exhibit similar decomposition patterns. The 23-kg pig is the most common pig model in such studies, but some researchers have used smaller pigs because they are inexpensive and more easily obtained. Although smaller pigs have been shown to adequately represent the local species composition dynamics throughout succession, they tend to attract fewer insects than larger carcasses and are thus more difficult to sample.

First, we compared the number and diversity of insects sampled by four sampling methods, two active (vacuum, sweep net) and two passive (emergence trap, sticky traps). We also compared their efficacy at different times of day. We found that no single method was able to outcompete the other methods during all decomposition days. The vacuum method collected the most species-rich samples, but sticky trap samples were the most diverse, when

both species richness and evenness were factored into a Shannon diversity index. Repeated sampling over the 12PM–6PM interval ensured that maximum diversity was sampled. All methods were deficient at sampling beetle species, so multiple sampling techniques must be integrated to effectively sample the community.

Second, we developed and optimized a new sampling method, the “vented-chamber.” The vented-chamber used thermal convection to direct attractive decomposition odors onto sticky traps, maximizing trap catches. This novel method collected significantly more necrophilous flies, representing a greater diversity, than the sweep net, the most common sampling method used in forensic entomology research and practice. The vented-chamber must be paired with hand collections to most effectively sample diversity, as the chamber alone trapped few beetles.

Third, we documented the ecological succession of insects arriving to neonate pig carcasses in central North Carolina during late summer. Six blow fly species were collected, as well as flesh flies and house flies. Ten beetle taxa were collected, including four species of scarab beetles. Fly activity overwhelmingly dominated the first 3 days of decomposition. By day 7, >50% of the pig carcasses were skeletonized and attracted few insects.

Fourth, we examined the effects of carrion relocation on the successional pattern of necrophilous insects. Pigs of various decomposition ages were relocated on the same day to a field that was ecologically similar to the field in which they decomposed. We examined the effects of decomposition age and relocation on necrophilous insect community assembly, while excluding non-olfactory cues. Olfactory cues alone differentially attracted insects and contributed to insect community separation by decomposition age. Insect community composition differed by decomposition age prior to relocation, but was not as pronounced

after relocation, although similar taxa and relative abundance of nearly all taxa were found on pigs both before and after transfer. The hairy maggot blow fly, *Chrysomya rufifacies*, was documented for the first time in central NC.

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Sampling and ecological succession of adult necrophilous insects

by  
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## **DEDICATION**

This work is dedicated to my grandparents, to whom I owe everything.

## **BIOGRAPHY**

Angela Marie Cruise (née Bucci) was born on January 9, 1989 in the town of Havre de Grace, Maryland and raised by her grandparents in Bel Air, Maryland.

Angela graduated with honors from Loyola University Maryland in 2011 with a degree in Biology. During her time at Loyola, she realized her love of the forensic sciences through undergraduate research experience in forensic entomology and an internship as an autopsy technician at the Office of the Chief Medical Examiner for the State of Maryland. A lifelong plan to attend medical school shifted to an interest in insects and forensics.

Angela joined Dr. Coby Schal's lab at NC State University in 2011. Soon after, she was awarded an NSF Graduate Research Fellowship that allowed her to develop her own project centered on forensic entomology. Angela has helped teach several courses at NC State and has done numerous guest lectures and outreach events both within and outside the entomology department.

Angela married her husband Casey in October 2015, and the two of them share a five year old black lab named Diesel.

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Thank you to my committee members for their guidance and feedback throughout grad school. You all have made me a better researcher.

Thank you to my fellow lab members, both past and present. I feel so lucky to have shared these years with you. When I moved to NC from MD, I worried about making friends in my new home. You all have gone above and beyond for me, and I can't tell you how much I appreciate our friendships. I couldn't have made it through grad school without all of your humor and advice. I would especially like to thank Rick Santangelo for all of your help these past 6 years. I couldn't have done half of these experiments (or used a table saw) without you. I'm going to miss your always positive attitude.

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## TABLE OF CONTENTS

LIST OF TABLES .....	vii
LIST OF FIGURES .....	viii
CHAPTER 1. Introduction.....	1
References.....	7
CHAPTER 2. Comparison of techniques for sampling adult necrophilous insects from pig carcasses .....	10
Abstract.....	11
Introduction.....	13
Materials and Methods.....	15
Results.....	19
Discussion.....	24
References.....	45
CHAPTER 3. A novel passive sampling technique for collecting adult necrophilous insects arriving at stillborn pig carcasses.....	49
Abstract.....	50
Introduction.....	51
Materials and Methods.....	54
Results and Discussion .....	61
References.....	80

CHAPTER 4. Ecological succession of adult necrophilous insects on neonate <i>Sus</i> <i>scrofa domesticus</i> in central North Carolina .....	85
Abstract .....	86
Introduction.....	87
Materials and Methods.....	89
Results.....	91
Discussion.....	95
References.....	122
CHAPTER 5. Effects of carrion relocation on the succession of necrophilous insects ..	126
Abstract.....	127
Introduction.....	128
Materials and Methods.....	131
Results.....	135
Discussion.....	140
References.....	157

## LIST OF TABLES

CHAPTER 2 .....	10
Table 1. List of forensically significant insects identified across all treatments .....	31
Table 2. Families and species of forensically significant insects trapped by each method .....	32
CHAPTER 3 .....	49
Table 1. List of forensically significant insects identified across all treatments .....	68
Table 2. Taxa sampled by the “vented-chamber” and sweep net methods.....	69
CHAPTER 4 .....	85
Table 1. List of forensically significant insects identified across all samples and their ecological associations.....	104
Table 2. Total abundance of each dipteran taxon across all pigs by day .....	105
Table 3. Total abundance of each coleopteran taxon across all pigs by day .....	106
CHAPTER 5 .....	126
Table 1. Placement and sampling schedule for all pigs .....	146
Table 2. Forensically significant insects sampled from all pigs and both experimental fields .....	147

## LIST OF FIGURES

CHAPTER 2 .....	10
Figure 1. Sampling and field setup .....	33
Figure 2. Modified Prokopack vacuum design .....	34
Figure 3. Emergence trap setup in the field .....	35
Figure 4. Sticky trap setup .....	36
Figure 5. Mean number of insects $\pm$ SEM trapped by each sampling method .....	38
Figure 6. Mean number of insects $\pm$ SEM trapped at each sampling time .....	39
Figure 7. Mean number of insects $\pm$ SEM trapped at each sampling time across decomposition days .....	40
Figure 8. Mean number of insects $\pm$ SEM trapped by each sampling method across decomposition days .....	41
Figure 9. Total abundance of each necrophilous insect taxon across all replicates.....	42
Figure 10. Relative percentage of each taxon trapped by each of the four sampling methods.....	43
Figure 11. Relative percentage of each taxon trapped at each sampling time .....	44
CHAPTER 3 .....	49
Figure 1. The “vented-chamber” trap. Expanded (A.) and assembled (B.) view .....	70
Figure 2. Wind roses for (A.) experiments 1–5 and (B.) experiment 6 .....	71
Figure 3. Effect of visual cue on mean number of insects $\pm$ SEM trapped .....	72

Figure 4. Effect of trap size and orientation on mean number of insects $\pm$ SEM trapped in each trap position facing West (A.) and East (B.).....	73
Figure 5. Effect of side openings on the number of insects trapped by the “vented-chamber”.....	75
Figure 6. Effect of chimney orientation on the number of insects trapped by the “vented-chamber”.....	77
Figure 7. Effect of increasing airflow on the number of insects trapped by the “vented-chamber” with different chimney orientations .....	79
CHAPTER 4 .....	85
Figure 1. Photograph (A.) and illustration (B.) of the “vented-chamber” method as outlined in Chapter 3.....	107
Figure 2. Environmental conditions during field experiments .....	108
Figure 3. Decomposition stages of neonate pigs .....	110
Figure 4. Number of pigs undergoing each of the five stages of decomposition throughout the seven days of the experiment.....	112
Figure 5. Mean number of (A.) taxa and (B.) insects sampled from pigs at each day of decomposition .....	113
Figure 6. Percentage representation of dipteran and coleopteran taxa trapped or hand-collected from pigs on each day of the 7-day decomposition process .....	114
Figure 7. Linear discriminant analysis of 8 neonate pigs over 7 days of decomposition .....	115

Figure 8. Percentage representation of mean number of dipteran individuals per taxon trapped or hand-collected daily from pigs during the 7-day decomposition process .....	116
Figure 9. Percentage representation of mean number of coleopteran individuals per taxon trapped or hand-collected daily from pigs during the 7-day decomposition process .....	118
Figure 10. Successional pattern of insects arriving to neonate piglets in central North Carolina in September 2015.....	120
CHAPTER 5 .....	126
Figure 1. Experimental fields at the Lake Wheeler Road Field Lab in Raleigh, NC ..	148
Figure 2. The “vented-chamber” method, as described in Chapter 3 .....	149
Figure 3. Daily minimum, maximum, and average ambient temperatures (°C) near the field research site during the experiment.....	150
Figure 4. Insect activity in the decomposition field (field 1) across all days of the experiment .....	151
Figure 5. Insect activity in the decomposition field (field 1) on the day of transfer, just prior to pig relocation .....	153
Figure 6. Insect activity in the transfer field (field 2) on the day of transfer, just after pig relocation.....	155

**CHAPTER 1.**

**Introduction**

Necrophilous, or “death-loving,” insects are attracted to carrion for feeding, breeding, predation, and/or shelter. These insects may be specialists who colonize the body or generalists who transiently visit a resource associated with the body (e.g., maggots or beetles for predatory insects, dung for dung-associated insects). Necrophilous insects are known to visit carrion in a predictable successional pattern [1-4], and the most prominent groups are from orders Diptera and Coleoptera. Insects that utilize carrion for feeding or breeding, such as the blow flies (Diptera: Calliphoridae) and flesh flies (Diptera: Sarcophagidae), arrive to a body within minutes of death to ovi- and larviposit [3, 4]. Their offspring feed on body tissues, assisting the decomposition process [5]. As the body progresses to later stages of decay, necrophilous beetles, primarily from orders Silphidae, Staphylinidae, and Histeridae, arrive and feed on both carrion tissues and other insects on the body, including both developing fly larvae and other beetles [6, 7].

Necrophilous insects have evolved finely tuned chemosensory systems to detect and compete for such transient resources [8, 9]. During the decomposition process, microbes produce dozens of volatile organic compounds (VOCs) that are attractive to both early responding flies and later arriving beetles [10-18]. The odor profile emanating from the decomposing body changes over time, as different body tissues are degraded [19-22]. Differential insect attraction to the changing volatile profile is believed to be a pivotal mechanism that drives predictable insect succession. The changes that a body undergoes in decomposition are classified by discrete stages. These stages (fresh, bloat, decay, postdecay/dry, and skeletal) describe the physical condition of the body over time (i.e.,

distended abdomen in the bloat stage or open body cavity in the decay stage) [7], but are not necessarily well correlated with arrival and departure patterns of necrophilous insects [23].

Because necrophilous insects arrive so quickly and predictably after death, forensic investigators have used their taxonomic identities and developmental stages, along with biotic and abiotic characteristics of the environment (e.g. plant cover, soil type, and temperature), to calculate the time since death, or the postmortem interval (PMI) [24]. These calculations are rooted in laboratory and field studies by forensic researchers to determine the growth rates of necrophilous insects at different temperatures and the effects of biotic and abiotic conditions on the succession of insects. In field studies, the most commonly used decomposition model is the 23-kg pig [25]. Pigs are used in place of human cadavers because they are easy to acquire and their decomposition pattern is similar to humans [25, 26]. Because of financial and logistical difficulties acquiring large pigs, however, some researchers have used smaller, often stillborn, pigs in their research. Smaller sized pigs have been shown to represent the local species composition dynamics throughout succession as well as larger pigs [27, 28].

In 2009, the National Research Council issued a report that widely criticized several forensic disciplines [29]. While forensic entomology was not directly addressed in the report, many of the NRC's criticisms of other disciplines likely apply to entomology research. Largely in response to this report, several forensic entomologists reviewed the literature and identified key areas of research that needed to be addressed [30, 31]. These suggestions included (a.) increased replication and standardization in ecological succession studies [32,

33]; (b.) increased documentation of geographical differences among insect populations and successional patterns, including those occurring on a microgeographic scale [34]; and (c.) more research on the proximate mechanisms by which necrophilous insects detect and are attracted to carrion [30].

In our efforts to address these key research areas, we used stillborn pigs as decomposition models. While the successional pattern of insects on neonate pigs remained as expected, these small pigs attracted fewer insects than larger carcasses. In Chapter 2, we examined the efficacy of four different methods for sampling neonate pigs. Two active (vacuum, sweep net) and two passive (emergence trap, sticky traps) were assessed at four different times of day throughout the decomposition process. No single method outcompeted the others across all decomposition days and the most common sampling method used in forensic entomology research and practice, the sweep net, was inadequate for gathering large samples from such small pigs. The most species-rich samples were collected by the vacuum method, but the most diverse samples were collected by the sticky traps, when both species richness and evenness were factored into a Shannon diversity index. None of the methods were able to effectively sample necrophilous beetles, so a combination of sampling techniques must be used in order to most effectively sample the entire necrophilous insect community. Additionally, several samples must be collected over the 12PM–6PM interval to ensure maximum diversity is represented. Our findings indicated that the vacuum and sticky traps were the most promising active and passive methods for sampling flies from neonate pigs. Modifications must be made to these methods, however, to maximize sample size.

In Chapter 3, we used our findings in Chapter 2 to develop a novel, passive sampling method. This method encloses a neonate pig in a sealed chamber and uses its thermally convected decomposition odors to direct insects to a pair of sticky traps placed atop a chimney on the chamber's lid. This method, called the "vented-chamber," outperformed the standard sweep net in sampling both fly abundance and diversity from neonate pigs. Its passive nature enables better standardization of insect sampling, and its inexpensive design allows researchers to increase replications with minimal effort and thus reduce reliance on pseudoreplication, a key concern posed by Tomberlin et al. [33] and Michaud et al. [32].

In Chapter 4, we documented ecological succession on stillborn pigs in central North Carolina during summer, using the vented-chamber. This investigation represents the first documentation of succession in this geographic location on neonate pigs and with the new sampling method. We collected six blow fly species, flesh flies, and house flies. Fly activity overwhelmingly dominated the first three days of decomposition. We also collected ten beetle taxa, including four species of scarab beetles, that remained in low numbers until day 4. Beetle activity dominated over fly activity on day 7, when over 50% of the pig carcasses were skeletonized.

Finally, we investigated the effect of carcass relocation on the successional pattern of insects visiting decomposing neonate pigs in Chapter 5. To do so, we simultaneously transferred pigs of four different decomposition ages to a new, but ecologically-similar field to that in which they decomposed. Insect communities were sampled with the vented-chamber and hand-collections in both fields. We found that insect community composition

differed by pig decomposition age in both fields, but that the differences were much more pronounced before relocation. Olfactory cues alone were sufficient to observe differential attraction by pig age. We also documented the hairy maggot blow fly, *Chrysomya rufifacies*, for the first time in central North Carolina. Our insights into the mechanisms of insect attraction and succession address one of the areas of needed research and are important contributions to the growing body of literature addressing the pre-colonization interval, or the period from death to insect colonization.

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**CHAPTER 2.**

**Comparison of techniques for sampling adult necrophilous insects from pig carcasses**

**Abstract**

1. Of particular need in forensic entomology are more studies examining the pre-colonization interval and mechanisms driving insect ecological succession. Methods of effectively sampling adult insects and knowledge of their diel and successional activity patterns are pivotal to ensure collection of the most abundant, species rich samples.
2. Stillborn pigs are excellent decomposition models because they are inexpensive and can be easily obtained, and therefore facilitate more replications and manipulation. Their small size, however, makes piglets much more difficult to sample than larger pig models.
3. Four sampling methods, two active (vacuum, aerial sweep net) and two passive (emergence trap, sticky traps), were compared on stillborn pigs in terms of the number of insects trapped and the diversity of samples. We also compared their efficacy at different times of the day to expose potential sampling biases.
4. Insects were identified and species counts were analyzed with generalized linear regression and ecological indices. Both method (NB glmm:  $\chi^2 = 20.693$ ,  $df = 3$ ,  $p < 0.001$ ) and time of day (NB glmm:  $\chi^2 = 29.814$ ,  $df = 3$ ,  $p < 0.001$ ) significantly affected the number of insects trapped from a pig. These factors were also each found to interact with decomposition day.
5. No single method was superior to the other methods during all three decomposition days. Sampling times after noon yielded the largest samples during the first two days of decomposition. On day 3, however, all sampling times were equally effective. Therefore, to

maximize insect collections from stillborn pigs, the method used to sample must vary by decomposition day.

6. The vacuum method collected the most species-rich samples. Sticky trap samples were the most diverse, when both species richness and evenness were factored into a Shannon diversity index. Repeated sampling over the noon to 6PM interval was the only way to ensure the maximum diversity of trapped insects.

7. The integration of multiple sampling techniques would most effectively sample the necrophilous insect community. All four tested methods were deficient at sampling beetle species. Therefore, future work should focus on optimizing the most promising methods, alone or in combinations, and incorporate hand-collections of beetles.

## **Introduction**

Forensic entomology links the science of entomology and the judicial system, most notably in death investigations. Forensic investigators use entomological evidence (e.g., insect species and developmental stages) from a putative crime scene and biotic and abiotic characteristics of the environment (e.g., plant cover, soil type, and temperature) to make inferences about the time since death, or the postmortem interval (PMI) [1]. Understanding these factors and other parameters such as geographic location and level of concealment enable the prediction of necrophilous insect species succession on a decomposing body, which is pivotal in PMI determination.

The characterization of ecological succession in general, and of necrophilous insects in particular, depends on accurate documentation of temporal and spatial changes in species diversity, richness, and abundance. For ephemeral ecological resources, such as small carcasses that are rapidly colonized by flying insects, the sampling methods used to collect newly arriving adults must be relatively unbiased and easy to implement without disrupting the successional process. Using an aerial sweep net (sweep net, henceforth) is the most common sampling method used in forensic entomology research and, in combination with hand-collections of larvae on and around the body, is the approved technique for investigators to collect necrophilous insects [2]. While this combination of methods adequately samples the fauna on large decomposing bodies, it has proven inadequate for succession ecology studies on small pigs [3]. Pigs are the most common models used in forensic studies in place of human cadavers because they are easy to acquire and decompose

much like humans [4, 5]. The 23-kg pig is the most common pig size used in such studies, but many researchers choose instead to use smaller pigs [5-7]. Although smaller sized pigs have been shown to adequately represent the local species composition dynamics throughout succession, they tend to attract fewer insects than larger carcasses [8, 9]. Sampling small carcasses such as stillborn pigs is challenging, because they represent small targets and arriving insects are readily disturbed by active sampling methods like sweep netting and do not readily return to such a small body. Nevertheless, stillborn pigs are easy to acquire and much less costly than larger pigs, so identifying less disruptive and unbiased sampling methods for documenting their associated fauna is critical.

Several passive sampling techniques, such as baited emergence traps, sticky traps (typically odor or tissue baited), and pitfall traps, have been used by researchers to sample necrophilous insects with fair success [3, 10-13]. The lack of direct and quantitative comparisons of active and passive sampling methods motivated us to use two active and two passive approaches to assess the diversity and number of insects trapped near stillborn pigs, as well as the relative ease of use of these methods. Sweep netting was chosen as one of the active methods because of its widespread use in forensic research and practice. Because no other active sampling methods could be found in the literature for sampling flying adult necrophilous insects, we adapted a vacuum targeting flying mosquitoes [14]. An emergence trap and sticky traps were selected as the two passive methods, with the whole pig itself serving as the bait, unlike in several previous studies that used only select tissues or synthetic odor blends [11, 12]. Of these methods, only sweep netting is commonly used in the practice

of forensic entomology, both by investigators at the crime scene and by researchers in the field [2, 15-17]. To our knowledge, none of the methods used in our investigation have ever been applied to the stillborn pig model. Additionally, we compared the diel pattern of trapping with the four methods to identify the optimal time of day for sampling to achieve maximum diversity and abundance.

## **Materials and Methods**

**Study site and experimental design** Experiments were conducted at North Carolina State University's Lake Wheeler Road Field Lab in Raleigh, NC (35.729310, -78.667451). Fully frozen stillborn pigs were acquired from the University's Swine Educational Unit. Freezing the pigs prior to field placement is not known to affect the successional pattern [18]. Because four sampling methods were compared concurrently, four pigs were used in each replicate of the experiment, with each pig assigned a different collection method (Figure 1a). Frozen pigs were placed in the field at 6AM on the day of the first sampling (Day 0). They were spaced 50 meters apart in full sunlight (Figure 1b) and were sampled four times daily – at 9AM, noon, 3PM, and 6PM – over a three day period to examine both the succession process and diel periodicity. The spacing between pigs ensured experimental independence of the pigs, and the full sunlight conditions reduced any potential variability caused by shading or vegetative differences at the four locations [19-21]. The experiment was replicated five times during June and July 2012.

**Sampling Methods** Two active and two passive insect sampling methods were used. A modified Prokopack aspirator (vacuum) [14] and sweep net were considered active collection methods, while an emergence trap and sticky traps were considered passive. All methods in this experiment focused on arriving adult necrophilous insects of orders Diptera and Coleoptera.

Sampling on all four pigs was performed simultaneously over a 10 min period. Passive sampling methods remained uninterrupted for the full 10 min time period. Preliminary work revealed, however, that both active methods were highly disruptive to insects at the pig body. Therefore, active sampling was performed for two alternating 1–1.5 min intervals on the pigs to allow insects to return to the pigs. Thus, vacuum sampling was conducted for 1–1.5 min on pig #1, then sweep net sampling was conducted on pig #2, and this process was repeated once during the 10 min interval, allowing disturbed insects to return to the pig. Meanwhile, passive sampling on pig #3 (emergence trap) and pig #4 (sticky traps) remained uninterrupted for 10 min.

***Modified Prokopack Vacuum*** The Prokopack aspirator was developed as a portable, cost-effective way to sample adult mosquitoes [14]. Like the original design, our vacuum featured an in-line blower motor, rubber coupling, and a DC battery (Figure 2). Unlike the Prokopack, our vacuum was not attached to a telescoping pole and did not have a built-in collection cup or backpack. Instead, wire mesh window screening was attached to a 6 cm long, 8 cm diameter section of PVC. The mesh allowed insects to be captured and prevented them from being pulled into the motor's blades. The PVC "trap" was connected to a 6.51 m<sup>3</sup>/min in-line

blower (Attwood® Turbo 4000, Item #1747-4) with a 10-to-8 cm rubber coupling. The blower was powered by a 12V 3ampHR sealed-electrolyte battery (XTREME®, XTAX4L-BS) with an in-line manual switch.

Sampling with the vacuum was limited to the area on and above the pig and did not include any local vegetation. At the end of each 1–1.5 min sampling interval, insects were transferred to a wide mouth (7.6 cm diameter) 473 mL mason jar containing 70% ethanol.

**Sweep Net** A 38 cm diameter sweep net with fine mesh netting was used to sample necrophilous insects. Aerial sweeping was performed in a zig zag motion with 180° twists as described by Wayne and Wallace [2]. As with vacuum sampling, sweep net sampling was limited to the area above the pig and did not include any sweeps of local vegetation. The contents from each 1–1.5 min of active sweeping were emptied into a mason jar containing 70% ethanol.

**Emergence Trap** A 96 x 26 mesh, 60 x 60 x 60 cm soil emergence trap (Bugdorm® Item #BT2003) was elevated 10 cm from the ground by stakes and placed over a decomposing pig (Figure 3), which allowed insects to enter the trap. Seventy-percent ethanol was used as the killing agent in the emergence trap's collection bottle. This passive trap was deployed over the pig for 10 min, after which it was removed and the collection bottle retrieved.

**Sticky Traps** Sticky traps were chosen as a passive method because of their documented use as successful baited traps [12, 22]. To maximize the surface area of the sticky traps, they were mounted on a wooden frame that allowed for six unscented insect monitoring sticky traps (LoLine®) to be simultaneously placed at several heights along the body of the pig

(Figure 4). To allow for easy placement and removal of the traps, cork stoppers were attached to the wooden frame with wood glue at various points, and traps were attached with standard pushpins (Figure 4). The frame with six sticky traps was placed over the pig and left undisturbed for 10 min. The traps were collected in plastic cling wrap.

**Identifications** Orders Diptera and Coleoptera were the main sampling targets, with emphasis on those necrophilous families commonly used in forensic entomology for PMI determinations. Because of their significant role as primary colonizers of decomposing bodies, the blow flies were further identified to species level using Whitworth's taxonomic key [23]. Insects on sticky traps were identified in situ.

**Analysis** Numbers of insects were analyzed to assess the effect of sampling method, date and time of day with negative binomial generalized linear mixed-effect models (NB glmm) with experimental blocks and individual pigs as random effects. Statistical analyses were performed in R using the lme4 package [24]. *P*-values of comparisons between treatment levels were calculated based on the *z*-distribution. Significance of each variable in the model was assessed by comparing models with and without respective variables or interactions using a  $\chi^2$  test [25].

Method and time of day were recorded for all samples. Diversity by method was assessed through overall counts by method, relative percentage of each taxon by method and time, and with three ecological indices. When considering diversity by method, we subdivided diversity into two components: species richness and species evenness [26]. Species richness refers to the number of forensically significant taxa collected respectively

by each of the four sampling methods [27]. Species richness (S) was represented by the number of taxa that were collected by each method across the five replicates. Species evenness is the consistency in the number of individuals across taxa. We calculated richness and evenness indices, as well as a Shannon diversity index which considers both richness and evenness as factors in its calculation [26, 28]. For the species evenness index and the Shannon diversity index, we included all taxa trapped within the community (experiment). Therefore, taxa that were not trapped at all by some methods were represented by zero. Formulas used to calculate evenness and Shannon diversity index values are as follows:

$$\text{Shannon index } (H') = - \sum_{i=1}^s p_i \ln p_i$$

$$\text{Evenness index } (J') = \frac{H'}{H'_{max}} = \frac{H'}{\log_e S}$$

## Results

**Taxa sampled** Only forensically relevant insects of the Orders Diptera and Coleoptera were included in the analyses, and calliphorids were further identified to species. Our collections included 7 calliphorid species (Table 1) that overlapped with collections by Cammack et al. [29], who also sampled in the same location.

**Effect of method and sampling time** Both sampling method and sampling time of day (indicated by “hour”) significantly affected the number of insects trapped (NB glmm, Method:  $\chi^2 = 20.693$ , df = 3, p < 0.001; Hour:  $\chi^2 = 29.814$ , df = 3, p < 0.001). However, the

interaction of these two factors was not significant (NB glmm,  $\chi^2 = 7.7301$ ,  $df = 9$ ,  $p = 0.5616$ ).

The emergence trap collected significantly fewer insects in total than the other three methods tested (Figure 5). There was no significant difference in the overall number of insects trapped by the vacuum, sweep net, or sticky traps; these methods were equally effective in terms of total insects sampled.

Sampling counts across all methods increased as the day progressed (Figure 6). Sampling at 6PM resulted in insect counts that were significantly higher than those collected at any other sampling time.

**Effect of decomposition day and sampling time** The total number of insects collected across all methods was significantly affected by the interaction of decomposition day and sampling time (NB glmm,  $\chi^2 = 41.434$ ,  $df = 6$ ,  $p < 0.001$ ; Figure 7). During the first two days of decomposition, afternoon sampling resulted in significantly larger samples than sampling at or before noon. On the second day of decomposition, this effect was continuous, with the number of insects trapped significantly increasing at each sampling time. The 6PM sampling time on this day produced the highest number of insects from any sampling time or day of decomposition. It is important to note that pigs were still thawing on day 1 of decomposition, so the ineffectiveness of early sampling times on this day may relate to the frozen nature of the pigs. Refrigeration is not known to alter the arrival times of insects, but the effect of freezing (without a thawing period) is unknown [18].

On the third day of decomposition, the time at which sampling was performed did not affect the overall number of insects trapped across methods; all four sampling times were equally effective for trapping insects.

**Effect of method and decomposition day** Day of decomposition had a significant effect on the number of insects trapped by method (NB glmm,  $\chi^2 = 20.262$ ,  $df = 6$ ,  $p < 0.01$ ; Figure 8). All sampling methods trapped their lowest respective number of insects on day 1 of decomposition. On this day, the sweep net and sticky trap methods were most effective, though the total numbers of insects trapped by these two methods were significantly lower than for any other day. The distinction among methods was less obvious on day 2 of decomposition, when the vacuum, sweep net, and sticky trap methods were equally effective for trapping insects. By day 3 of decomposition, clear differences between methods could be seen, with the vacuum trapping more than double the number of insects of any other method.

The sampling method most influenced by decomposition day was the vacuum method. This method's efficacy over decomposition greatly varied in comparison to the other methods. On decomposition day 1, the vacuum and the emergence trap methods trapped the smallest number of insects. By day 2 of decomposition, the vacuum method, sweep net, and sticky traps were most effective. On day 3, the vacuum significantly surpassed any of the other methods for number of insects trapped. All other methods remained steady in their rankings on days 2 and 3, whereas the vacuum's efficacy increased on each successive day.

**Taxonomic patterns by sampling method** Insects were compared across methods (Figure 9) and by relative abundance of each taxon by method (Figure 10). Calliphoridae was the

most abundant family trapped, followed by Sarcophagidae (Figure 9). The five most abundant insect taxa (*Lucilia illustris*, Sarcophagidae, *Phormia regina*, *Lucilia coeruleiviridis*, *Cochliomyia macellaria*), four of which are calliphorids, were trapped by all four sampling methods, but few beetles were trapped by each of the four methods (Figure 10).

Species richness was highest with the vacuum method, with a total of 13 taxa trapped, followed by sticky traps (10 taxa) (Table 2). The vacuum method trapped at least one insect from each taxon, with the exception of staphylinid beetles (Table 2). It was the only method that captured *Lucilia cuprina* and *Chrysomya megacephala*, which were the rarest calliphorid species trapped overall (Figure 8). Additionally, the vacuum was the only method that trapped representatives from the coleopteran families Silphidae and Dermestidae. The traditional sweep net method captured 8 taxa, including all calliphorids except the two rarest species; but it only captured representatives of one beetle family, Histeridae (Table 2). The emergence trap captured only 5 taxa and failed to capture the three least abundant calliphorid species and any beetles (Table 2); it was also the only method that did not trap *Musca domestica*. Sticky traps captured all calliphorid species except for the two rarest, and it also trapped species in three beetle families (Table 2). It was the only method that trapped staphylinid beetles, which, along with histerid beetles, were the most abundant beetle families trapped overall across all methods. Overall, none of the methods were able to collect all beetle taxa, and only the vacuum method captured all fly taxa (Table 2).

**Taxonomic patterns by diel periodicity** No taxa were unique to the 9AM sampling time, but several taxa were unique to each of the other sampling times (Figure 11). Dermestid beetles and *C. megacephala*, a rare blow fly in this study, were trapped only at noon. *L. cuprina*, another rare blow fly in this study, was trapped only at 3PM, and *Necrophila americana*, a rare beetle, was trapped only at 6PM. Several other taxa were trapped during two of the four sampling times, including histrid beetles at 12PM and 3PM, *Lucilia sericata* at 12PM and 6PM, and scarab beetles at 3PM and 6PM. *M. domestica* was trapped at all sampling times except 9AM.

**Diversity indices** Species evenness is a measure of each species' numerical representation in the community. Given the relatively small number of insects trapped per species, with some species not represented at all in some sampling methods, this index may be strongly affected by both highly and poorly represented species. Indeed, the emergence trap, which had the poorest taxonomic representation had the highest evenness index ( $J' = 0.782$ ). The relative percentages of each taxon trapped by method are shown in Figure 10.

The Shannon index is an overall diversity index that considers both species evenness and species richness, with larger values representing greater diversity. Shannon index values indicated that the sticky trap collections were the most diverse ( $H' = 1.786$ ), followed by the vacuum method ( $H' = 1.700$ ). Emergence trap collections were the least diverse ( $H' = 1.258$ ).

## **Discussion**

A National Research Council 2009 report criticized forensic disciplines from odontology to genetics, recommending more sound basic biological research to strengthen their scientific foundations and increase the credibility of findings in the court system [30]. In response, forensic entomologists have begun to reframe research within more ecological and evolutionary genetics frameworks [31, 32]. Tomberlin et al. stressed the importance of examining the biological and ecological mechanisms driving necrophilous insect attraction and succession, as well as other aspects of the pre-colonization interval [31]. Additionally, forensic entomologists stressed a need to better document ecological succession patterns and growth rates of necrophilous insects in various geographic locations to better describe the inherent variation [33-35]. Pivotal to all these endeavors is efficient sampling of the necrophilous arthropod community.

**Sampling Method.** The vacuum, sweep net, and sticky trap methods trapped statistically equal amounts of insects in total across all days and times. This overall ranking did not hold true throughout the decomposition process, however; the ranking of methods varied by decomposition day, a rough approximation of PMI. Thus, experiments designed to maximize collections possible each day would need to consider switching the method used to sample insects over day of decomposition or use multiple methods. The sweep net or sticky trap methods were most effective on the first day of decomposition. On this day, the pig did not attract many insects, especially when compared to subsequent days of decomposition. The two active methods as well as the sticky traps were equally effective on day 2, and the

vacuum was the most effective method on day 3. The emergence trap method trapped fewer insects than the other methods, both in an overall method analysis and in each of the three decomposition days. This method should not be used when large insect samples are required.

When examining the diversity of samples, the vacuum and sticky trap methods outcompeted the other methods. The vacuum was the only method that trapped all nine fly taxa in this study. Flies are the most common insects used in PMI determinations, so accurately documenting their diversity, and especially first arrival, on the body is of extreme importance [1, 5]. The sweep net and sticky trap failed to trap the two rarest fly species, (*Lucilia cuprina* and *Chrysomya megacephala*) indicating that perhaps these methods could be improved to be more effective—the sweep net by altering the motion so that it is less disruptive and the sticky traps by better utilizing the pig's decomposition odors, known factors in blow fly host finding [11, 36]. The vacuum method outcompeted the other methods for sampling fly diversity, but the sticky traps were more effective for sampling overall diversity, as indicated by Shannon indices.

Beetle diversity proved much more difficult to document in terms of both overall number and diversity. The vacuum method trapped the greatest beetle diversity, four of the five taxa, with the sticky trap method collecting three of the five beetle taxa, including the Staphylinidae, the only family exclusively trapped by this method. The insufficiency of all selected methods for trapping beetles echoes Schoenly et al.'s findings when assessing various methods on larger, 23–27 kg pigs—aerial and sticky trap sampling mostly target

flies, with hand collections or pitfall trapping as better methods for sampling beetles [3].

Either hand collections or pitfall trapping should be included in a sampling protocol.

From a qualitative perspective, it is important to note that the two most promising methods for documenting diversity, the vacuum and sticky traps, were damaging to the insect specimens they collected. Others have found similar damage when using sticky traps with necrophilous insects [37]. The vacuum method greatly damaged many of the insects it trapped. Taxonomic identifications of many necrophilous insects, especially flies, depend on setae patterns and antennal characteristics. These characters were often lost or damaged in the vacuum samples. Very small insects were especially damaged, and some were even small enough to fit through the window screening mesh attached to the vacuum. Design modifications, such as using nylon stockings as the collection vessel, should remedy these shortcomings of the vacuum trap. While insects could not be removed from sticky traps, despite several attempts with various oils and solvents, insects were not damaged to the point that they could not be identified. Almost all flies landed on the trap such that characteristic setae on the wings and body were undamaged and visible. If insects do not need to be removed, the use of sticky traps is not problematic, although these traps are cumbersome to transport and store.

Both active methods were disruptive to insects on the pig body, so multiple samples should always be taken when using either of these methods to ensure that all target insects are captured. The passive methods offered unbiased, non-disruptive collections. These

methods can collect insects for any chosen duration. Their insect collections can be readily compared between trials or experimenters since experimental bias is minimal.

**Diel periodicity** In general, sampling later in the day (i.e. after noon) yielded larger samples than in the morning. The only day where this was not true was day 3, when all sampling times were statistically equivalent in terms of number of insects trapped. In a repeated sampling design with a consistent sampling time each day, after noon sampling should be performed, as it will ensure the largest samples across decomposition days. Such a design, however, only targets large samples and does not consider diversity of samples.

Identifying an ideal sampling time by both overall abundance and diversity is challenging and depends greatly on experimental design. While the afternoon times resulted in larger overall samples, specific taxa trapped varied greatly with time, both at and after noon. Though its samples were smaller than those collected after noon, the noon sampling time was the only period during which dermestid beetles and *C. megacephala*, a rare blow fly in this experiment were captured. Other rare species, including *L. cuprina* and *N. americana* were collected at 3PM and 6PM, respectively. In light of these differences over time, it is difficult to pinpoint one ideal sampling time. Repeated sampling during the interval from 12PM to 6PM would account for all observed diversity, as no unique species were found prior to 12PM. Sampling after noon would also help ensure that samples are large.

We used relatively short 15 minute sampling intervals in our design, so the latest sampling time, 6PM, was feasible. This may not be possible in experiments that use a long sampling interval. Nevertheless, our results highlight that large numbers of insects are

trapped close to dusk, even though many species are not active at night [38, 39]. The length of the sampling interval and season should be considered when sampling late in the day.

Repeated sampling of communities is critical for estimates of ecological indices and succession. The goal of this work was to identify sampling methods from stillborn pig carcasses that would result in large and species rich samples. We found significant differences in the number of insects trapped by method, time of day, and day of decomposition. The sampling protocol and sampling time depend on the goals of the study. Clearly, a combination of sampling methods deployed for long sampling durations is most desirable. However, most studies are constrained by limited manpower and time. Moreover, sampling for long durations might interfere with the decomposition process and ecological succession.

Several general ideas emerged from our analysis:

1. Studies seeking to maximize abundance of necrophilous insects over decomposition should sample in late afternoon (in mid-summer), but a combination of sampling methods should be used to sample over days of decomposition because the effectiveness of methods changes over time.

In ecological succession studies focusing on diversity over time, the vacuum method should be used each day, but sampling should be repeated several times between noon and dusk. When manpower is limited, the sticky traps performed well at trapping diversity, although it should be noted that they are difficult to store, and it is difficult

- to isolate voucher specimens. Nevertheless, in combination with periodic use of the vacuum trap or sweep net this sampling design would be effective.
2. Sweep netting, the established methodology for sampling large pigs, was effective at sampling insects. Notably, however, it was less effective at representing the local species diversity. The need to sample for maximal diversity is highlighted by the observed significance of individual species in the decomposition process, independently of their abundance [40]. With several modifications (see above), the vacuum method, which captured the greatest species richness from the pigs, might be more effective than the forensic standard: the sweep net.

Sampling of beetles was ineffective in this study. An important goal of future work should be to improve sampling of beetles. Both hand collections and pitfall traps (active and passive sampling methods, respectively) are labor-intensive. Zanetti et al. found that combining both methods yielded abundant beetle samples with less bias for certain taxa over others [41]. Perhaps ground-touching sticky traps should be considered.

### **Acknowledgements**

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Table 1: List of forensically significant insects identified across all treatments. Families are in bold with species identified listed below. If no species listed, taxonomic identifications ended at the family-level.

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Forensically significant insects identified
Diptera
<b>Calliphoridae</b>
<i>Lucilia illustris</i>
<i>Lucilia coeruleiviridis</i>
<i>Lucilia sericata</i>
<i>Lucilia cuprina</i>
<i>Phormia regina</i>
<i>Cochliomyia macellaria</i>
<i>Chrysomya megacephala</i>
<b>Sarcophagidae</b>
<b>Muscidae</b>
<i>Musca domestica</i>
Coleoptera
<b>Histeridae</b>
<b>Silphidae</b>
<i>Necrophila americana</i>
<b>Staphylinidae</b>
<b>Scarabaeidae</b>

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Table 2. Families and species of forensically significant insects trapped by each method.

	Diptera							Coleoptera						
	Calliphoridae							Sarcophagidae	Muscidae	Histeridae	Scarabaeidae	Staphylinidae	Silphidae	Dermestidae
	<i>Lucilia illustris</i>	<i>Phormia regina</i>	<i>Lucilia coeruleiviridis</i>	<i>Cochliomyia macellaria</i>	<i>Lucilia sericata</i>	<i>Lucilia cuprina</i>	<i>Chrysomya megacephala</i>		<i>Musca domestica</i>				<i>Necrophila americana</i>	
Vacuum	•	•	•	•	•	•	•	•	•	•		•	•	
Sweep	•	•	•	•	•			•	•	•				
Emergence trap	•	•	•	•				•						
Sticky traps	•	•	•	•	•			•	•	•	•			

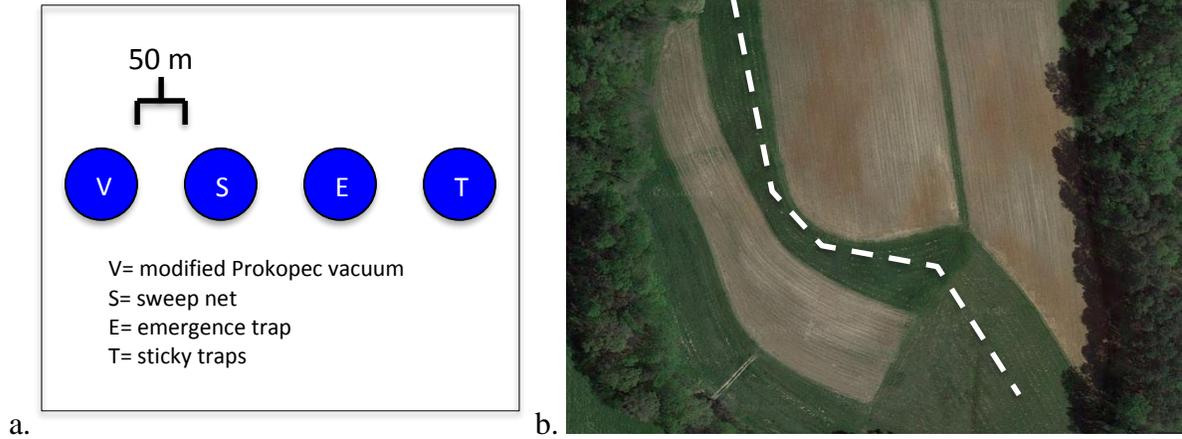


Figure 1. (a) Pigs, represented by the blue circles, were placed 50 m apart with each assigned a sampling method. This ordered pattern of methods was conserved across replicates. (b) Aerial overview of field site at the Lake Wheeler Road Field Lab in Raleigh, NC. Pigs were placed 50 m apart along the dotted line across all replicates.

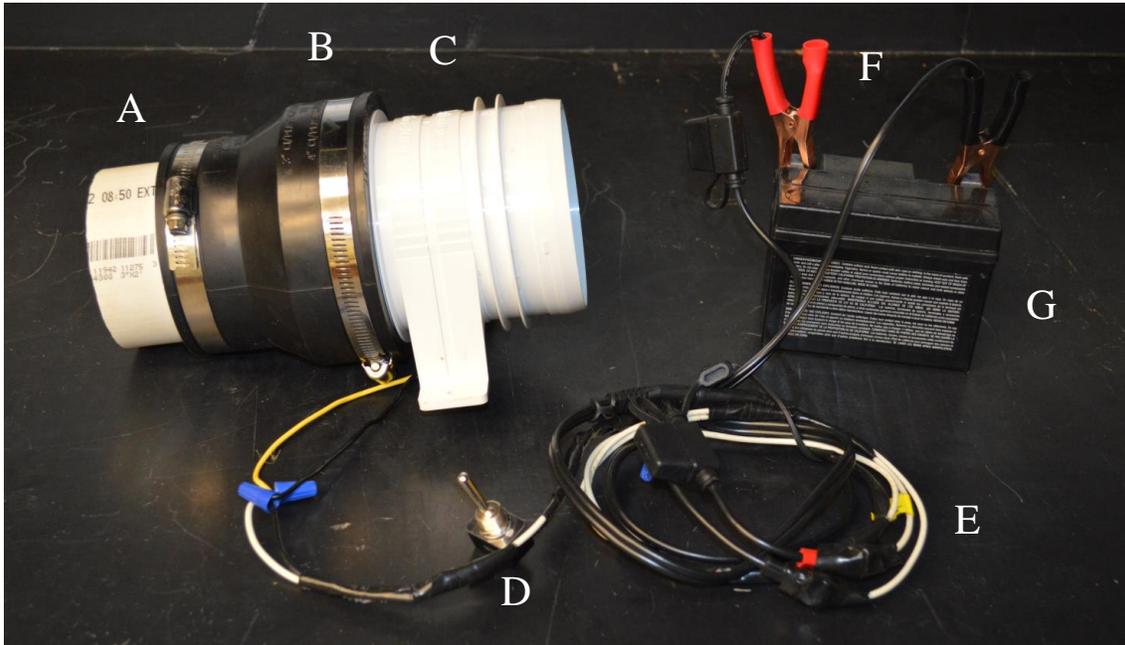


Figure 2. Modified Prokopack vacuum unit. (A) 8 cm diameter PVC. (B) 10-to-8 cm rubber coupling. (C) In-line blower motor. (D) In-line on/off switch. (E) Additional wiring. (F) Positive and negative battery clamps. (G) 12V 3ampHR battery.



Figure 3. Emergence trap setup in the field. The emergence trap was elevated four inches off the ground to ensure insect arrival was not interrupted.

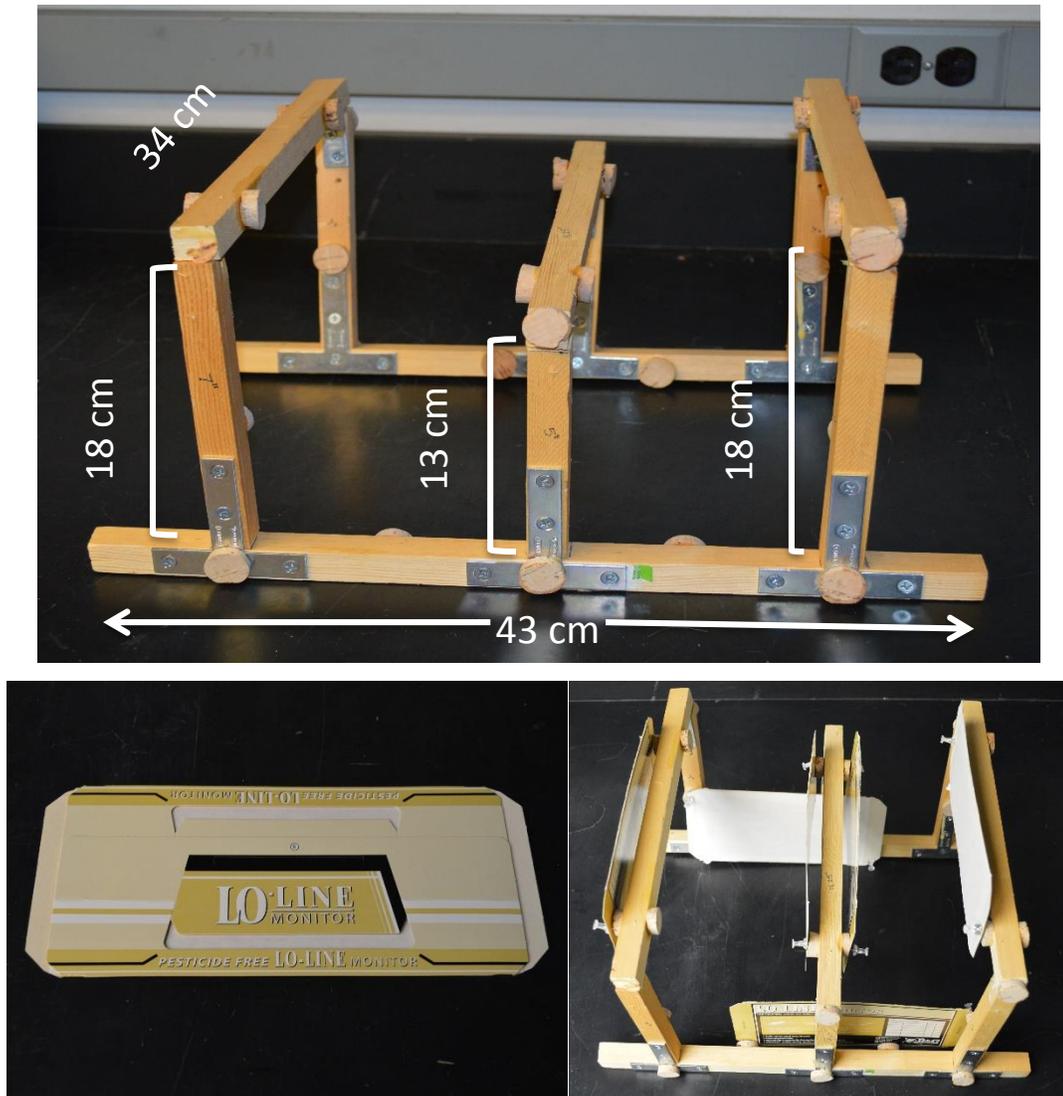


Figure 4. (top) Wooden frame marked with dimensions. The frame was placed over the pig lengthwise, such that the two 18 cm arms were over the pig's head and tail ends, respectively. Corks were glued to the frame and served as attachment points for the sticky traps. (bottom left) LoLine<sup>®</sup> sticky trap. These traps are designed with two paper attachments that can be joined together to create a tent-like structure. These attachments were removed

from all traps prior to their connection to the wooden frame. (bottom right) Wooden frame with sticky traps attached. The six traps were each attached with two push pins.

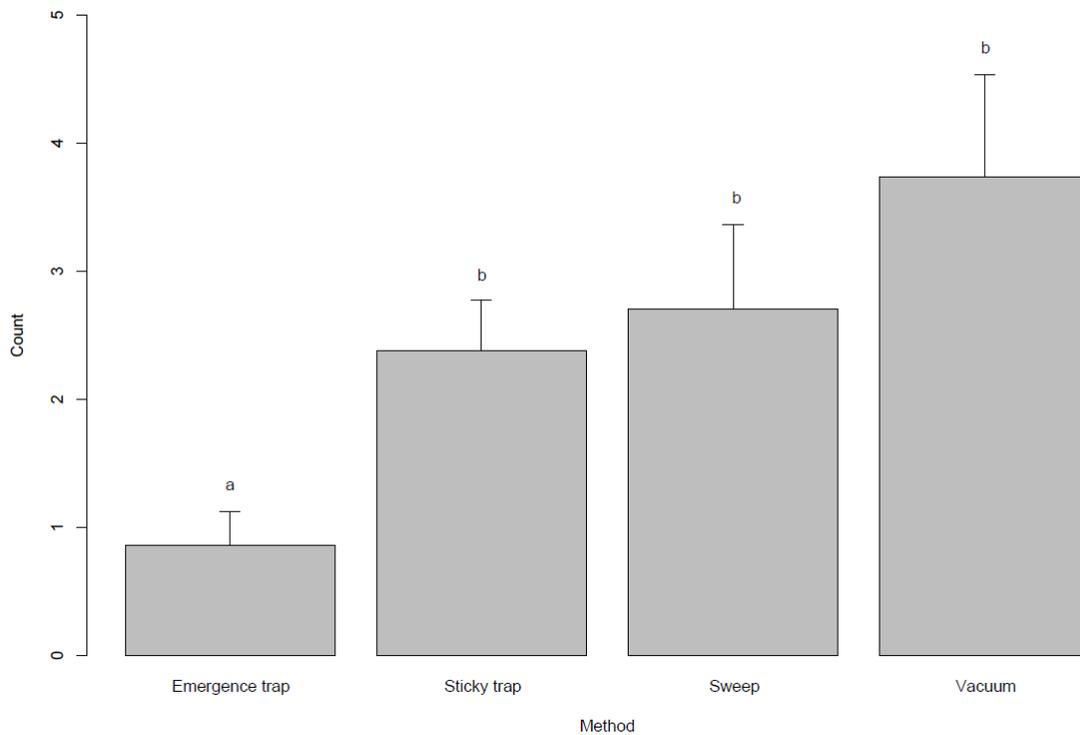


Figure 5. Number of insects trapped by each sampling method (NB glmm,  $\chi^2 = 20.693$ ,  $df = 3$ ,  $p < 0.001$ ). Bars show mean values  $\pm$  SE. Methods labeled with the same letter are not significantly different ( $p < 0.05$ ).

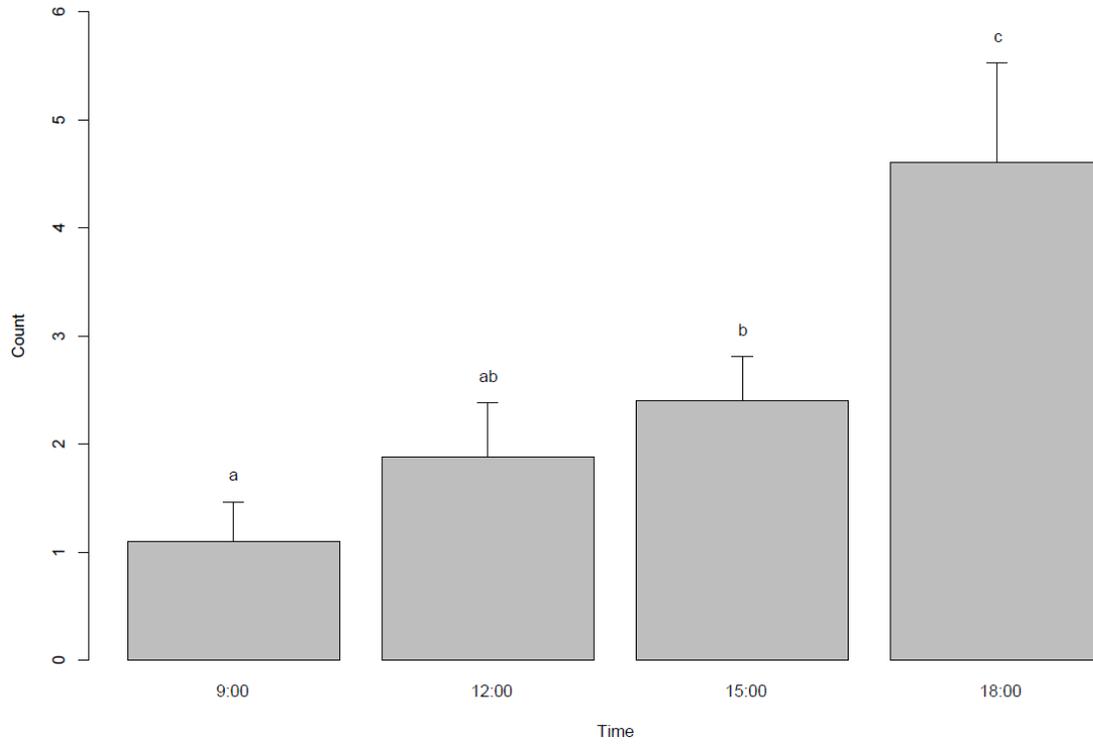


Figure 6. Number of insects trapped at each sampling time (NB glmm,  $\chi^2 = 29.814$ ,  $df = 3$ ,  $p < 0.001$ ). Bars show mean values  $\pm$  SE. Bars labeled with the same letter are not significantly different ( $p < 0.05$ ).

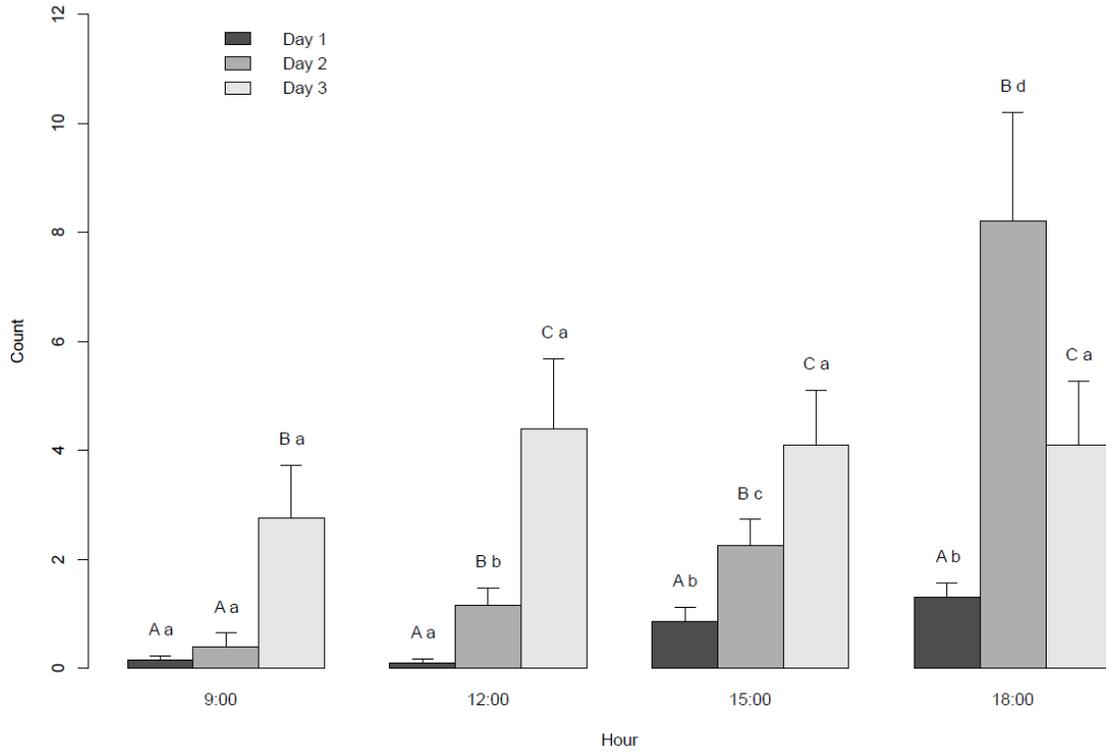


Figure 7. Number of insects trapped at each sampling time along days (NB glmm,  $\chi^2 = 41.434$ ,  $df = 6$ ,  $p < 0.001$ ). Bars show mean values  $\pm$  SE. Bars labeled with the same upper and lower case letter are not significantly different within the same hour and day, respectively ( $p < 0.05$ ).

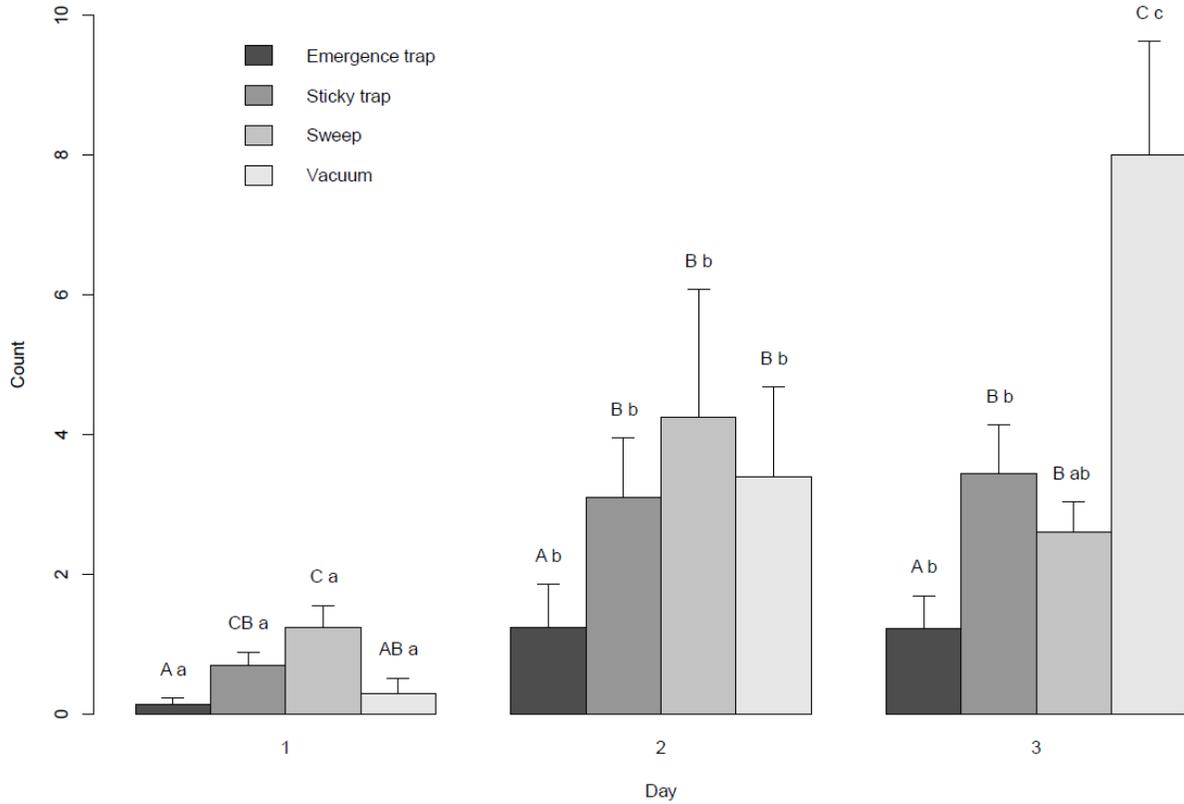


Figure 8. Number of insects trapped by each sampling method along days (NB glmm,  $\chi^2 = 20.262$ ,  $df = 6$ ,  $p < 0.01$ ). Bars show mean values  $\pm$  SE. Bars labeled with the same upper and lower case letter are not significantly different within the same day and method, respectively ( $p < 0.05$ ).

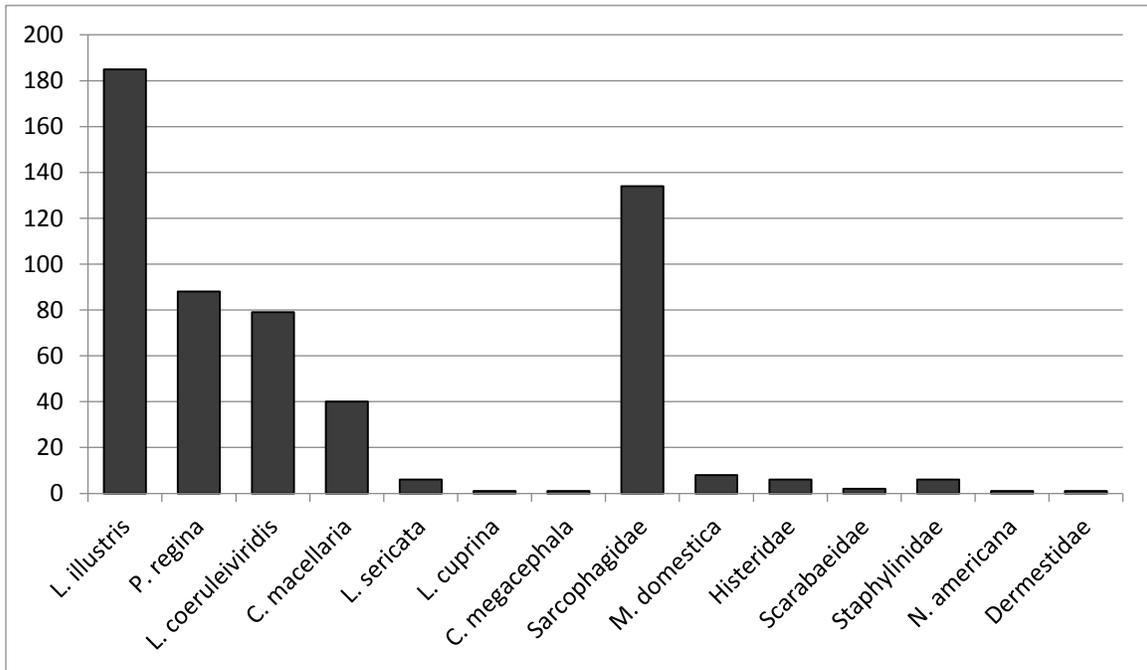


Figure 9. Total abundance of each necrophilous insect taxon trapped across all replicates. Fly taxa were trapped in much higher numbers than beetle taxa.

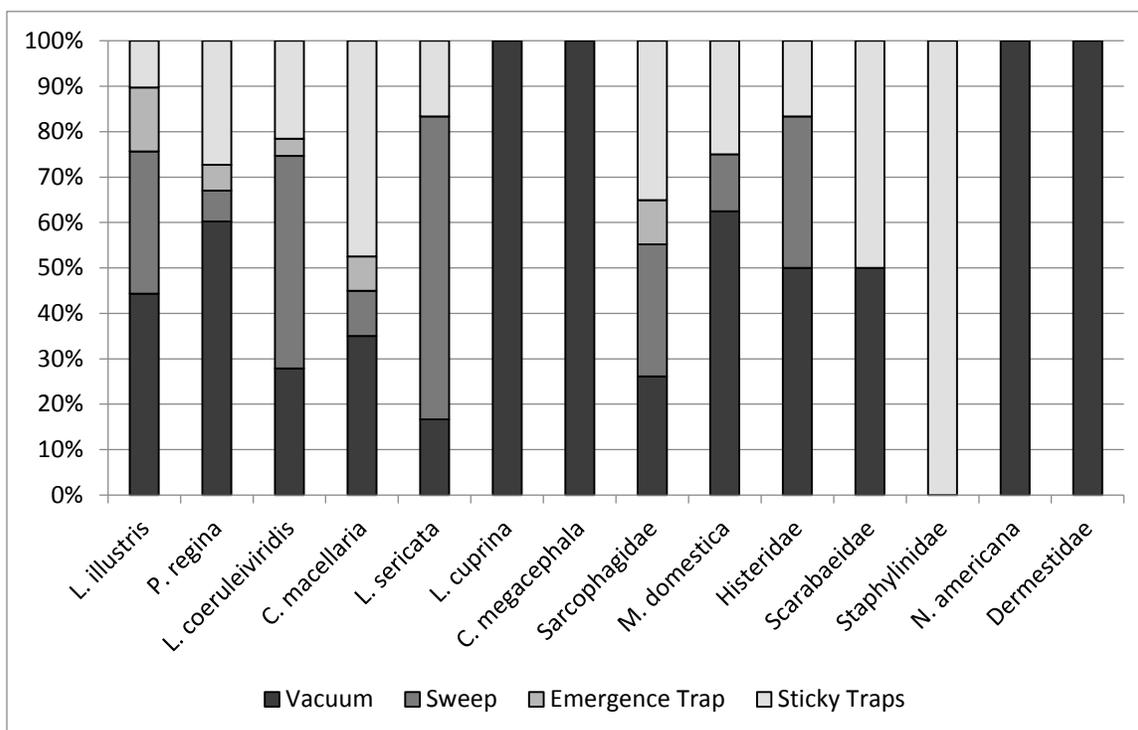


Figure 10. Relative percentage of each taxon trapped by each of the four sampling methods. The five most abundant taxa (*L. illustris*, Sarcophagidae, *P. regina*, *L. coeruleiviridis*, and *C. macellaria*) were sampled with all four methods. Several rare taxa, including *L. cuprina*, *C. megacephala*, *N. americana*, and Dermestidae were trapped only with the vacuum method. Staphylinid beetles were only sampled with the sticky traps.

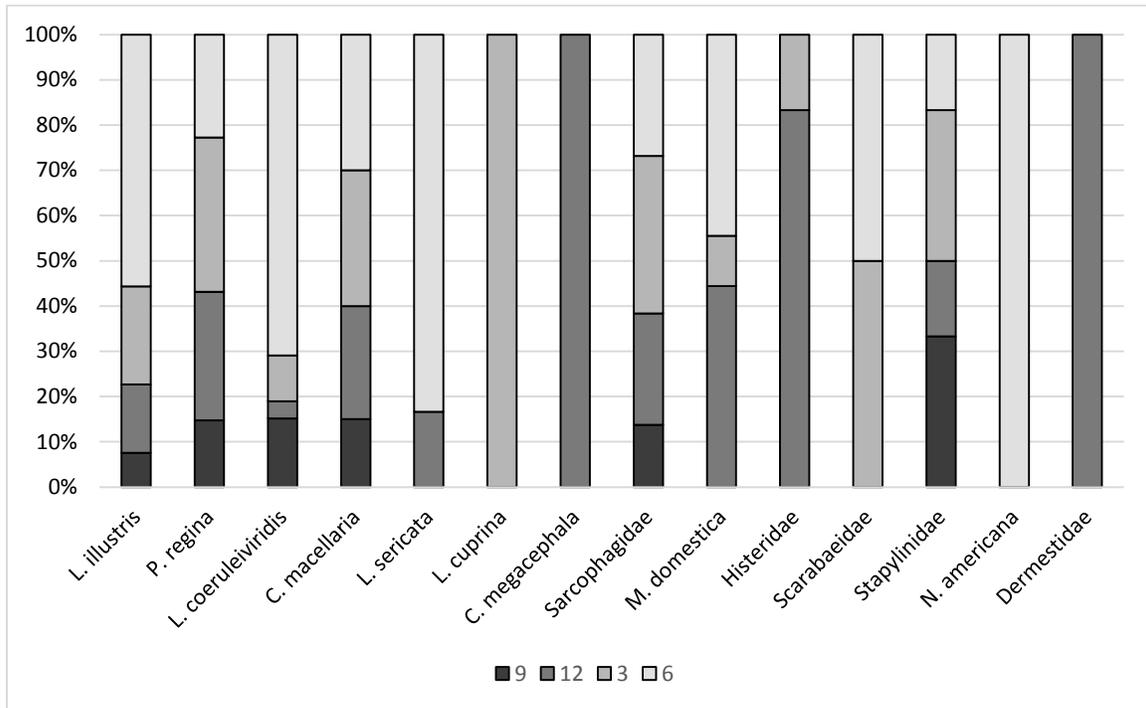


Figure 11. Relative percentage of each insect taxon trapped at each sampling time. No unique species were trapped before noon.

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**CHAPTER 3.**

**A novel passive sampling technique for collecting adult necrophilous insects arriving at  
stillborn pig carcasses**

**Abstract**

1. Neonate pigs are established decomposition models that can be easily acquired for experimental studies in forensic entomology. Their small size, however, poses challenges to traditional sampling methods of necrophilous insects, like sweep netting, the most commonly used sampling method in forensic entomology research and practice.
2. Previous research experimentally demonstrated the potential for sticky traps as an effective sampling method for collecting necrophilous insects from stillborn pigs. While this method effectively sampled fly diversity from the pigs, it shared with sweep netting low sample diversity and abundance, particularly of necrophilous beetles.
3. Motivated by chemosensory host-finding of necrophilous insects and the architecture of carrion-mimicking thermogenic flowers, a “vented-chamber” method was developed and its design experimentally optimized. In this approach, a neonate pig was transiently enclosed in a chamber. The heat produced by the decomposition process thermally convected the natural decomposition odors in the headspace above the pig toward a pair of sticky traps.
4. Pigs were sampled with both the “vented-chamber” method and sweep net. The “vented-chamber” method collected significantly more necrophilous flies, representing a greater diversity than the sweep net ( $p=0.0018$ ).

5. The “vented-chamber” approach used thermal convection to direct attractive odors onto sticky traps and thus guided necrophilous insects to the traps to maximize trap catches. Nevertheless, this approach caught few beetles, and hand collections must be paired with it to most effectively sample beetle diversity.

## **Introduction**

Necrophilous insects from orders Diptera and Coleoptera are important in the field of forensic entomology, as their arrival patterns and growth rates can be used to calculate a post-mortem interval, or PMI [1]. In death investigations, a PMI is used to estimate a person’s time of death, potentially corroborating or contradicting an alibi [2]. A dead body, whether carrion or human, is a rare and ephemeral resource in the ecological landscape [3, 4]. Therefore necrophilous insects, including both specialists and generalists, have evolved specialized, fine-tuned, chemosensory systems to detect and compete for such transient resources [5, 6]. A central tenet of PMI determination is that necrophilous insects rapidly locate and colonize a carcass in a predictable ecological succession [7-10]. Blow flies (Diptera: Calliphoridae) and flesh flies (Diptera: Sarcophagidae) are typical first responders and have been shown to arrive to a body within minutes of death [9, 10]. Thus, the period of insect activity (PIA), which can be estimated using growth tables and local temperatures, is closely related to the PMI [11].

Experimental research in forensic entomology requires the use of animal models. Neonate stillborn pigs have been used as decomposition models in a number of forensic

studies, and their low cost and ease of acquisition allows researchers to increase their number of replications and reduce reliance on pseudoreplication, thus addressing a major criticism of forensic entomology research [12-14]. However, while carcass size has little effect on the successional pattern of necrophilous insects, it significantly influences the overall number of insects attracted to the carcass, challenging the effectiveness of typical sampling procedures [15, 16]. The combination of sweep netting and hand-collecting has been demonstrated experimentally to representatively sample the fauna colonizing a decomposing body [17] and serves as the standard practice for crime scene investigators. This approach is significantly constrained, however, by small collections of insects, which is further exacerbated by the disturbance caused by sweep netting over a small carcass [18]. Therefore, for active sampling methods like sweep netting to be effective, multiple, short, interrupted sampling events are required at each replicate body, which may limit the number of replicates that can be sampled concurrently. This is further exacerbated if insect sampling needs to be coupled with other intensive biological and environmental sampling.

Previous research on the suitability of sampling methods for ecological succession studies using neonate pigs showed that passive sticky traps effectively sampled arriving insects (Chapter 2). Indeed, collecting necrophilous insects with sticky traps is not a new technique; such traps are frequently baited with natural or synthetic odors for blow fly monitoring or trapping purposes [19, 20]. Unique to our previous work, however, was that the decomposing pig served as bait, with sticky traps mounted to an open wooden frame placed above it. The sticky traps sampled a diversity of flies, but they were relatively

ineffective at sampling beetles, and it was clear that necrophilous insects were adept at orienting to the carcass while avoiding the traps (Chapter 2). Thus, the efficiency of sticky traps was depressed by the spatial separation of olfactory cues from the decomposing pig and the position of the sticky traps.

In the present work, we sought to increase the efficiency of sticky traps by directing the decomposition odors to the sticky traps and guiding the arriving insects to this attractive target. Our “vented-chamber” method was inspired by two observations: (1) necrophilous insects quickly locate hosts primarily through orientation to decomposition odors [20-24], and (2) carrion-mimicking plants attract necrophilous insect pollinators with a combination of odors, heat, and visual cues to guide the insect to the flower opening [25-28].

The initial activation, orientation, and landing behaviors of necrophilous insects rely heavily on odor cues [20, 29, 30]. As a body decomposes, microbes proliferate and metabolically produce dozens of microbial volatile organic compounds (MVOCs) [31-33]. These compounds, particularly nitrogen-containing compounds (e.g. indole), short chain alcohols (e.g. nonanol), acids (e.g. butanoic acid), and sulfur-rich compounds (e.g. dimethyl trisulfide (DMTS)), are attractive to necrophilous insects, including both early responding flies and later arriving beetles [21, 26, 29, 34-36]. Several of these MVOCs have also been used to develop synthetic odor blends for blow fly monitoring and trapping purposes [20, 34, 37]. Carrion-mimicking plants like the dead horse arum exploit this reliance on host odors in necrophilous insects by producing sulfur-rich volatiles that attract necrophilous insect pollinators [26, 38]. Such plants are often thermogenic, where heat facilitates odor emission

and thermotactic orientation by pollinators, and they include visual cues which guide insects to the flowers [25, 27, 28].

Similarly, we developed a way to thermally convect natural decomposition odors to attract necrophilous insects and guide them to a sticky trap target. The “vented-chamber” design takes advantage of heat produced during the decomposition process to thermally convect natural pig decomposition odors through a chimney system leading to a pair of sticky traps. This passive trap design maximized trap catch with minimal active sampling by researchers. We experimentally assessed the effects of trap design features on trap catch.

## **Materials and Methods**

**Overview** All experiments were performed at North Carolina State University’s Lake Wheeler Road Field Lab in Raleigh, NC (35.728713, -78.666719) in June-August 2015 and June-July 2016.

**Pig Placement** Fully frozen stillborn pigs (*Sus scrofa domesticus*) were acquired from the University’s Swine Educational Unit and remained in a freezer until placement in the field for all experiments. The “vented-chamber” method required that the pig be temporarily placed within a chamber during sampling for necrophilous insects. To facilitate this, each pig was placed on a layer of soil on standard 35 x 46 cm cafeteria trays. Ten minutes before pigs were placed in the field, soil from the site was piled atop the cafeteria tray to a height of ~ 4 cm. A pig was placed on the soil-covered tray on the ground. This arrangement allowed us to

move the decomposing pigs into the chamber for sampling and ensured that there was still a soil/body interface for maggot and beetle activity [4, 39].

**Trap Design** The design for the “vented-chamber” trap was inspired by preliminary field observations, necrophilous insect host-finding mechanisms, and the pollination strategies of carrion-mimicking thermogenic flowers. The basic design, which served as our positive control, consisted of a watertight 41.2 quart (39 liters) plastic tub (#10054386, The Container Store, Coppell, Texas) with locking airtight lid and several PVC adapters (Figure 1A). A stillborn pig placed on a 4 cm layer of soil on a cafeteria tray was placed in the chamber, and natural pig decomposition odors served to attract adult flies and beetles to the trap. The chamber had three openings: one at the center of each of the small (45 cm) sides of the tub, which allowed ambient air to enter the chamber, and one offset on one side of the lid, which led to a PVC “chimney” (Figure 1B). Within each of these holes was a 5 cm internal diameter PVC coupling fitting. This fitting allowed for different lengths of PVC to be inserted at each port for easy, non-permanent experimental manipulation. The PVC chimney focused the odors through a small opening, guiding arriving insects to the sticky traps. In the basic design, the side opening near the pig’s head was capped, forcing ambient air to enter the chamber through the opposite side and vent the headspace through the chimney. All experiments were conducted with one sticky trap facing west and the other facing east. The prevailing winds at the Lake Wheeler Rd. Field Lab were from the Southwest for both the 2015 and 2016 experiments (Figure 2).

**Sampling Protocol** Before sampling, a pig on a cafeteria tray was placed into the tub with the pig's head orientated toward the capped end (Figure 1B). The lid was then secured in place, ensuring that the PVC chimney was closest to the capped end of the tub. Two unscented Super Catchmaster<sup>®</sup> glue traps (Catchmaster, AP&G Co, Inc., Bayonne, New Jersey) were attached back-to-back with a binder clip and inserted into the slits of the chimney coupling. The “vented-chamber” was allowed to passively sample arriving insects for 15 min. The traps were then covered with plastic cling wrap and stored at -80°C until insect identification. The pig was removed from the chamber and placed on the ground for 15 min to minimize disruption of its natural colonization and also prevent excessive heating within the chamber which might stress the maggots [40]. For all experiments, sampling occurred between noon and 6PM (Chapter 2).

**Experiment 1: Visual vs. olfactory cues** The sticky traps on the “vented-chamber” might serve as visual cues, and insects alighting on the traps might contribute to trap catch. Three frozen pigs were placed 25 m apart at the field edge in partial shade and allowed to decompose for 24 hrs. Each pig was then exposed to two treatments in a randomized order: a positive “vented-chamber” control (Figure 1B) and a no-chamber experimental treatment (Figure 3). For the latter, a metal ring stand set adjacent to the pig held a PVC chimney with two back-to-back sticky traps (Figure 3). The chimney was set at the same height and position over the pig as in the “vented-chamber” control. Each of the two treatments was assayed for 15 min, with a 15 min rest period on the ground in between, during which the chamber and ring stand were removed. The three pigs were simultaneously sampled three

times daily for two consecutive days. This experiment was performed in July 2015 and repeated with two additional pigs.

**Experiment 2: Trap size** Odors from the decomposing pig were directed toward either two back-to-back glue traps (Figure 1B) or an array of 12 back-to-back traps that formed a larger target (Figure 4). The large trap consisted of two back-to-back 41 x 41 cm white poster boards held together with binder clips, each covered with six glue traps (Figure 4 schematics). This large trap was supported between two 1-m tall rebar rods and oriented to face west. A frozen stillborn pig was placed atop a soil-covered cafeteria tray and allowed to decompose for 48 hrs. The 2-day decomposing pig was then sampled for 15 min with the two-trap method, removed from the chamber to aerate on the ground for 15 min, and sampled again for 15 min with the 12-trap array. This experiment was repeated with four additional pigs in June 2015, for a total of five replicates, and the order in which the two trap types were performed was randomized across replicates. The overall numbers of necrophilous insects were compared, and their spatial distribution on the array of the 12 traps was assessed.

**Experiment 3: Number of chamber openings** In this air flow experiment, we altered the number of sides open and closed on the chamber. The use of PVC couplings and caps on the “vented-chamber” allowed for easy, non-permanent manipulation of air flow. Two fully frozen pigs were placed 25 m apart in a partially shaded location at the field edge in June 2015. Pigs were sampled for 15 min with each of three randomized treatments: one side open, both sides open, both sides closed (Figure 5). All sampling began 24–48 hrs after pig

placement in the field, and each pig received each treatment one time. This experiment was replicated with four additional pigs in July 2015.

**Experiment 4: Chimney orientation** In preliminary observations, we noticed that traps placed on a vertical chimney atop the chamber caught more insects than traps attached to a horizontal chimney on the chamber's side. Eight pigs were used in this experiment, with each pig exposed twice to each of four treatments, as detailed in Figure 6. This design maintained the position of the trap either above the chamber or at its side, while varying the origin of the chimney between a position above the side vent hole or in line with it. These arrangements were expected to drive different thermal convection forces within the “vented-chamber”. Treatment order was randomized for each pig, and sampling was performed for 15 min with a 15 min rest period on the ground between sampling periods. Pigs were spaced 25 m apart in partial shade in July 2015. Sampling began 48 hours after pig placement in the field.

**Experiment 5: Passive vs. forced venting of the headspace** A 92 x 92 x 25 mm SilenX Effizio<sup>®</sup> computer fan with an air flow of 42 ft<sup>3</sup>/min (71m<sup>3</sup>/hr) was used because of its low noise (15dBA) and portability (12VDC power source). The fan was attached to the 8 cm end of an 8-to-5 cm piece of PVC coupling with epoxy putty. This coupling was then joined to the 6 cm piece of PVC on the open mesh end of the chamber (Figure 7). Four frozen pigs were placed 25 m apart on soil covered cafeteria trays in the field in August 2015 and allowed to decompose for 48 hrs. The pigs were then sampled for 15 min with each of the four chimney orientations with the fan-modified “vented-chamber”. Chimney orientations were randomized, and each orientation was repeated twice with each pig. As in all other

experiments, the pigs were placed on bare ground for 15 min between successive 15 min sampling periods. This experiment was repeated with four additional pigs.

**Experiment 6: “Vented-chamber” vs. sweep net** Two frozen pigs were placed 50 m apart at the field edge in partial shade and allowed to decompose for 48 hrs. Two sampling treatments were performed twice on each pig: sweep netting and the standard “vented-chamber” sampling. As before, successive 15 min sampling periods were separated by a 15 min rest period with the pig placed on the ground. The order of the two treatments was randomized, and sampling was repeated the following day. This experiment was repeated once more with two pigs and a final time with a single pig in June and July 2016. Insects trapped by the sweep net were killed and stored in 70% ethanol. The numbers and species composition of sticky trap and sweep net catches were assessed.

**Identifications** The plastic wrap was removed from sticky traps while the adhesive was still frozen. Traps were then thawed for 10–15 min, and insects were identified in situ, without removing them from the traps. Insects trapped by sweep netting were removed from ethanol, allowed to dry, and identified.

Insects were first classified as to whether or not they were forensically significant. These insects are known to feed or breed on carrion and are useful to forensic investigations and PMI determination; literature documenting local fauna of interest was also consulted [41, 42]. Forensically significant insect taxa collected in these experiments were documented (Table 1). For Experiments 1–5, insects were counted without further classification. For

Experiment 6, insects were classified to family or species (Table 1). Calliphorids were identified to species using Whitworth's taxonomic key [43].

**Analyses** Two-tailed paired t-tests were used to assess the effect of visual cues (Experiment 1), insect trap size (Experiment 2), and comparison of the “vented-chamber” vs. sweep net (Experiment 6) on the number of insects trapped ( $\alpha = 0.05$ ). Differences in fly numbers trapped in each region of the large trap (Experiment 2) were further analyzed with one-way ANOVA followed by Tukey's HSD test (SAS 9.4, SAS Institute Inc.). To assess the effect of the number of openings on the number of insects trapped (Experiment 3), a generalized linear model (GLM) was fit on insect count, with a Poisson error distribution and a logarithmic link function, using the statistical procedure GLIMMIX (SAS 9.4, SAS Institute Inc.) [44]. All pigs were subjected to each experimental condition ( $n = 6$  per experimental condition) and considered a random effect factor. Pairwise least squares means comparisons, with a Tukey's Kramer adjustment to control for Type I Error (reject null hypothesis of no effect when null hypothesis is true), were used to assess significant differences in the number of insects trapped by experimental condition ( $\alpha = 0.05$ ) [45].

Examination of the effects of trap orientation both with (Experiment 5) and without (Experiment 4) the addition of a fan was performed much like the analysis described for the number of sides open, except that both pig and a pig \* orientation interaction were included as random effects in both analyses to better account for outliers present in these datasets.

Means are reported  $\pm$  SEM for all experiments.

## Results and Discussion

**The vented-chamber** We designed the “vented-chamber” in response to consistently small samples collected from neonate pig carcasses. The “vented-chamber” consisted of an airtight chamber into which a decomposing pig was placed during sampling times. Ports on the sides of the chamber allow for manipulation of air flow, and a chimney at the top of the chamber opens to a set of sticky traps for insect collection. This design dramatically increased the number of trapped necrophilous insects by directing the odors from the decomposing pig to a set of sticky traps.

**Visual vs. olfactory cues** White colored traps, like those used in the “vented-chamber”, are typically neither attractive nor repellent to various types of blow flies, but may increase fly alightment on the trap surface [29, 46, 47]. In experiment 1, insects did not use the sticky traps as visual cues and were adept at orienting to the decomposing pig. Whereas the standard “vented-chamber” trap captured  $18.4 \pm 4.37$  insects, the no-chamber sticky trap did not trap any insects (Figure 3; paired t-test:  $T = 4.211$ ,  $df = 9$ ,  $p = 0.0011$ ). These results also underscore the importance of the chamber and chimney in directing decomposition odors toward the trap.

Wall and Fisher also found higher trap catches when a white target was coupled with odor, but the white target itself was not attractive to flies [48]. Other studies seeking to optimize traps for blow flies of veterinary significance have shown that certain colors like yellow, when added to an odor baited sticky trap, may increase trap catch [29, 49-52]. Interestingly, blow flies approaching an odor source responded to vertically-oriented visual

cues (contrast) to initiate a landing response, but not to horizontally-oriented cues [37].

Further experiments altering trap color of the “vented-chamber” could help to better describe the roles of olfactory and visual cues at close range. For example, painting a black target on the vertically-mounted sticky trap may increase fly landing efficiency. Our focus, however, was to assess this method for ecological succession studies, and thus keeping the traps as neutral as possible was a priority.

**Trap Size** In Experiment 2, trap size did not affect the number of insects trapped. Although the square area of the large trap was six times larger than the standard small trap, both trapped similar mean numbers of insects (large trap:  $35 \pm 1.29$  insects; standard trap:  $42.8 \pm 14.31$  insects; paired t-test:  $T = 0.44$ ,  $df = 4$ ,  $p = 0.6587$ ). Thus, from a practical perspective, adding extra surface area with more sticky traps is unwarranted given the additional cost of the traps and effort to secure them.

Moreover, the grouping of insects at the region of the chimney opening was a clear indication of the importance of odors in attraction, as well as the success of the chimney itself as a way of directing necrophilous insects to a particular location for trapping.

On the large trap, significantly more insects were trapped on the sticky trap positioned just above the chimney than on any other trap position (West-facing:  $F_{5,30} = 9.45$ ,  $p < 0.0001$ ; East-facing:  $F_{5,30} = 7.99$ ,  $p < 0.0001$ ; Figure 4). In total, 85.1% of the insects were trapped directly above the PVC chimney, indicating that insects used mainly olfactory cues and that the traps were rarely used as visual cues or resting sites. Significantly greater trap catch on the East-facing traps of the large trap (paired t-test;  $T = 2.44$ ,  $df = 5$ ,  $p = 0.03$ )

is consistent with the predominance of winds from the West Southwest during these assays (Figure 2). Olfactory navigation in flying insects (positive anemotaxis) involves upwind navigation, so the East-facing traps would be encountered first by newly arriving insects [53].

**Number of chamber openings** In Experiment 3, presence of at least one opening to the outside air significantly affected the number of insects trapped by the “vented-chamber” (glmm:  $F_{2,10} = 12.44$ ,  $p = 0.0019$ ; Figure 5). Chambers with one or two openings trapped significantly more insects ( $22.9 \pm 11.39$  insects and  $23.11 \pm 11.49$  insects, respectively) than chambers with both sides closed ( $14.13 \pm 7.07$  insects). These results indicate that decomposition odors diffuse through the chimney even when there is no air exchange within the chamber. However, the addition of another opening in the chamber allows outside air to enter the chamber and facilitates thermal convection of the decomposition odors.

**Chimney orientation** As predicted by our preliminary observations, chimney orientation in Experiment 4 significantly affected the number of insects trapped (glmm:  $F_{3,21} = 6.72$ ,  $p = 0.0024$ ; Figure 6). The top ( $16.82 \pm 5.12$  insects) and angled top ( $16.52 \pm 5.06$  insects) orientations trapped significantly more insects than the angled side ( $6.06 \pm 2.09$  insects) and side ( $5.78 \pm 1.86$ ) treatments. These results support the notion that thermal convection is an important mechanism in the “vented-chamber” design. Orientations where the chimney originated at the top of the box (top, angled top) trapped significantly more insects than orientations where the chimney originated from the side of the box (side, angled side).

These results are likely attributed to decomposition MVOCs thermally convecting from the pig. Heat is released during the decomposition process, and it is expected to be vented through a chimney, especially when cooler fresh air is allowed to displace the escaping air [54]. Heat serves not only to transport MVOCs, but heat itself is a known cue for blow flies at close range, contributing to its effect in the “vented-chamber” trap [25].

**Passive vs. forced venting of the headspace** Experiment 5 investigated whether the addition of a fan would overcome deficiencies of some treatments in Experiment 4 by annulling the effects of differential passive thermal convection. Indeed, the forced discharge of the headspace with a fan equalized the insect counts from all chimney orientations (glmm:  $F_{3,21} = 0.23$ ,  $p = 0.8717$ ; Figure 7). However, the fan added additional cost and maintenance but did not significantly increase trap catches relative to our standard design. These results support our evidence that the decomposition odors play a pivotal function in host location by necrophilous insects, and the location of the entry to the body (i.e. sticky trap position) is of minor significance as long as odors efficiently emanate from that position.

**“Vented-chamber” vs. sweep net** In paired comparisons of the passive “vented-chamber” and active sweep netting (Experiment 6), the former method captured significantly more insects than the sweep net ( $34.13 \pm 9.74$  insects vs.  $7.08 \pm 1.79$  insects; paired t-test,  $T = 3.237$ ,  $df = 23$ ,  $p = 0.0018$ ). Both methods sampled seven common taxa, including most blow flies, flesh flies, and the house fly (Table 2). Flesh flies (sarcophagids) were trapped in a larger abundance as well as in a greater percentage of sweep net samples, but all other flies were trapped in larger numbers and in more samples in the “vented-chamber” method. Only

the “vented-chamber” method trapped the blow fly *Lucilia cuprina*, and only the sweep net method trapped a single histerid beetle.

**Advantages and disadvantages of the “vented-chamber”** An ideal sampling method should be easy to implement, unbiased, and cost-effective, while allowing the researcher to sample maximum diversity and overall numbers of organisms. We systematically modified the “vented-chamber” to maximize trap catch. Results of all design modifications indicated that the prototype trap, i.e. the positive control, was the most effective design for trapping the largest number of insects. These modifications, however, were useful to understand which features of the trap design contribute most to its efficacy.

The “vented-chamber” is an effective method for trapping a relatively large number of necrophilous insects as they arrive to the carcass. Its advantages are low cost, portability, and the ability to trap large and diverse insect communities. This passive trap facilitates sampling from multiple carcasses at once, with minimal input from the researcher. The “vented-chamber” trap also eliminates sampler bias, which is almost unavoidable when active sampling methods are used in field research. It significantly increased trap catch over previous work with sticky traps alone, and our results demonstrated that without the chamber, the sticky traps were completely ineffective. Thus, a key feature of this chamber that contributes to its effectiveness is the venting of decomposition odors onto a sticky trap.

Nevertheless, a major shortcoming of the “vented-chamber” trap is that it, like most sampling methods, failed to trap necrophilous beetles. We frequently observed that necrophilous beetles flew at low altitude to an area near an exposed pig and then walked to

the resource; some beetles hovered just centimeters above the carcass and then dropped onto it. This would explain why beetles were not found on the elevated traps. A modification of the “vented-chamber” could place the sticky traps in contact with the ground, possibly in combination with a fan to broadcast the decomposition odors close to the ground. It is possible that even these modifications may not trap beetles because beetles will likely be deterred from stepping onto the sticky surface. We postulate, however, that this modified, fan-assisted “vented-chamber” approach could focus beetles into a small area on the ground from which they will be easier to hand collect. This is an acceptable approach, as a combination of active and passive methods has been shown to most effectively sample necrophilous beetle diversity [55]. In forensic entomology practice and succession research, sweep netting is almost always coupled with hand-collections [7, 17, 56-59].

Our findings have implications for forensic entomology. First, by incorporating knowledge about odor, thermal, and visual cues used by necrophilous insects in navigation to carcasses, we developed a passive trap that maximized trap catches from small decomposing bodies. This simple, inexpensive “vented-chamber” design was a substantial improvement over the open frame sticky trap design used in our previous work (Chapter 2). Additionally, and perhaps more importantly, the “vented-chamber” trapped significantly more insects than sweep netting. This novel sampling method could be readily adapted by forensic investigators to rapidly establish reference collections of local fauna using neonate pig carcasses or other carcasses. There are obvious constraints scaling this method to larger bodies. We suspect, however, that tent-like devices with open bottoms, similar to emergence

traps, could be used instead of chambers. Greater heat and convection might facilitate MVOC transport toward the sticky traps. Notably, larger collections of necrophilous insects throughout the carcasses' decomposition will provide greater resolution of the differential attraction of males, unmated females, and mated females to the carcass.

A second implication is the simple observation that more necrophilous insects are attracted to an odor source when it is passively or actively “vented”. Thus, a decomposing corpse within a “chamber” with only one opening (e.g., a bag) may emit substantially less decomposition odors (and attract fewer insects) than when at least two openings are present (e.g., rolled up carpet). The latter would allow for faster air exchange and odor emission due to convective processes.

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Table 1. List of forensically significant insects identified across all treatments. Families are in bold with species identified listed below. If no species is listed, taxonomic identifications ended at the family-level.

Order	Family	Genus and species
Diptera	Calliphoridae	<i>Lucilia illustris</i> (Meigen)
		<i>Lucilia coeruleiviridis</i> (Macquart)
		<i>Lucilia sericata</i> (Meigen)
		<i>Lucilia cuprina</i> (Wiedemann)
		<i>Phormia regina</i> (Meigen)
		<i>Cochliomyia macellaria</i> (Fabricius)
Coleoptera	Sarcophagidae	Sarcophagidae spp.
	Muscidae	<i>Musca domestica</i> (Linnaeus)
	Histeridae	Histeridae spp.

Table 2. Taxa sampled by the “vented-chamber” and sweep net methods.

	“Vented-chamber” trap		Sweep net	
	Number trapped (mean $\pm$ SEM)	Percentage positive (N = 24)	Number trapped (mean $\pm$ SEM)	Percentage positive (N = 24)
<i>L. illustris</i>	4.92 $\pm$ 1.17	62.5%	0.71 $\pm$ 0.18	50.0%
<i>L. coeruleiviridis</i>	2.88 $\pm$ 0.99	50%	0.92 $\pm$ 0.32	41.7%
<i>L. sericata</i>	1.46 $\pm$ 0.43	45.83%	0.29 $\pm$ 0.13	20.8%
<i>L. cuprina</i>	0.17 $\pm$ 0.10	12.5%	0	0
<i>P. regina</i>	22.29 $\pm$ 8.09	87.5%	3.50 $\pm$ 1.13	66.7%
<i>C. macellaria</i>	0.71 $\pm$ 0.32	29.17%	0.21 $\pm$ 0.10	16.7%
Sarcophagidae spp.	0.38 $\pm$ 0.17	20.83%	0.96 $\pm$ 0.28	54.2%
<i>M. domestica</i>	0.88 $\pm$ 0.38	37.5%	0.42 $\pm$ 0.19	25.0%
Histeridae spp.	0	0	0.04 $\pm$ 0.04	4.2%
<b>Total</b>	33.69 $\pm$ 11.65		7.05 $\pm$ 2.37	

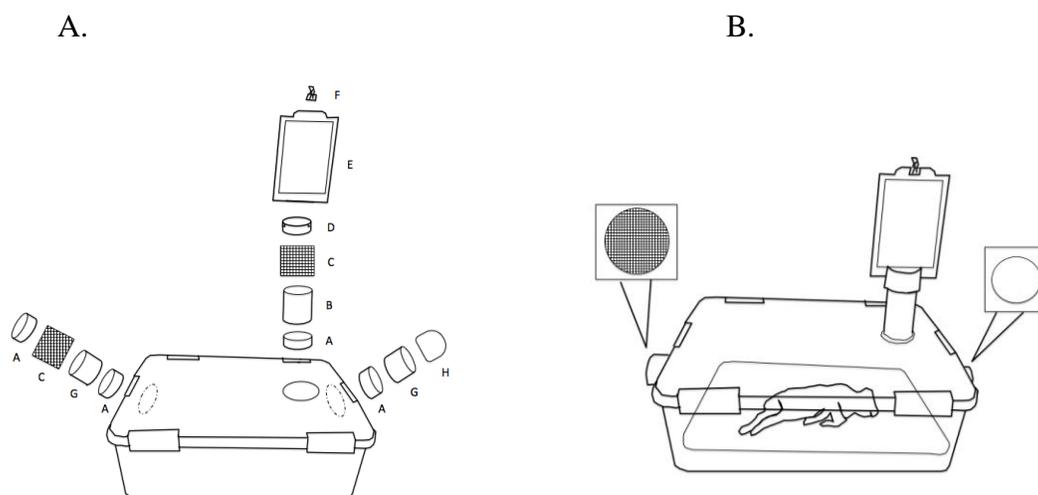
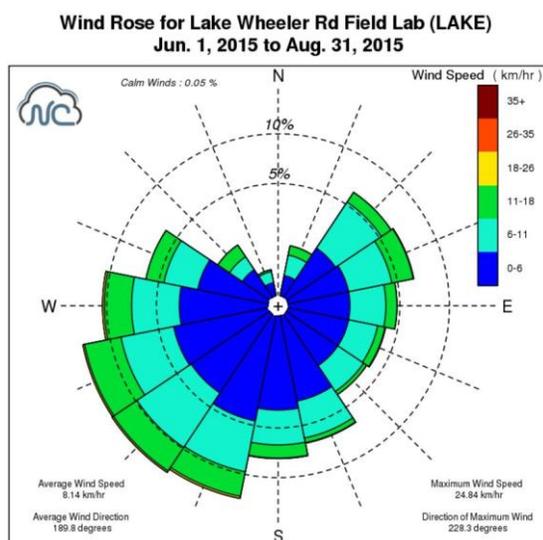


Figure 1. The “vented-chamber” trap. Expanded (A.) and assembled (B.) view. (A) 5 cm PVC coupling; (B) 14 cm section of 5 cm diameter PVC; (C) 10 x 10 cm square of 17 x 13 mesh vinyl-coated polyester window screening; (D) 5 cm diameter PVC coupling with two slits for holding glue traps; (E) Back-to-back unscented Super Catchmaster<sup>®</sup> glue traps; (F) Binder clip. (G) 6 cm PVC section of 5 cm diameter PVC. (H) 5 cm diameter PVC end cap. A pig was placed on a cafeteria tray within the chamber. The left side of the chamber contained a mesh-covered opening, whereas the right side was capped. In all experiments, a pig was placed in the chamber as illustrated, with its head closest to the capped side and chimney. This setup served as the positive control for most experiments.

A.



B.

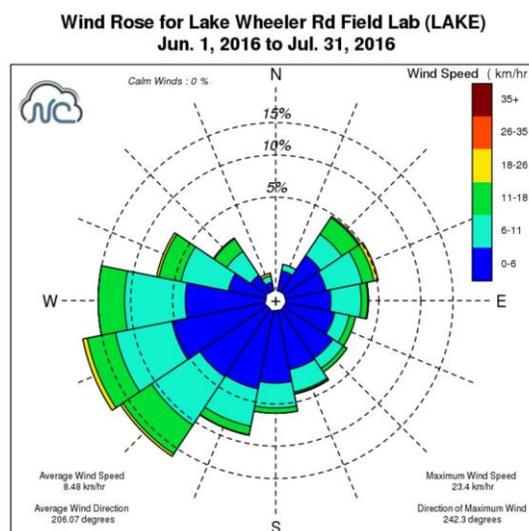


Figure 2. Wind roses for (A.) experiments 1–5 (June – August 2015) and (B.) experiment 6 (June – July 2016). The predominant wind direction was from the West Southwest. All data from the State Climate Office of North Carolina Lake Wheeler Rd. Field Station (<http://climate.ncsu.edu/windrose.php?state=NC&station=LAKE>).

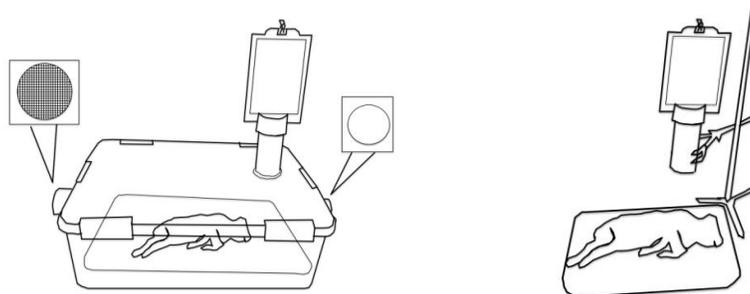
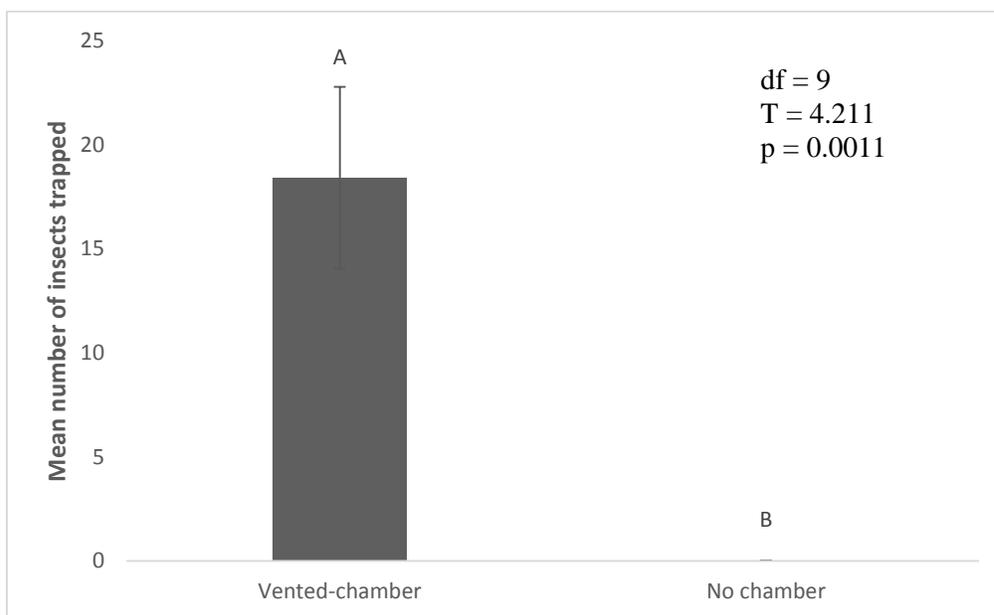


Figure 3. Effect of visual cue on mean number of insects ( $\pm$  SEM) trapped. The “vented-chamber” trapped significantly more insects than the no-chamber treatment, which failed to trap any insects (paired t-test:  $T = 4.211$ ,  $df = 9$ ,  $p = 0.0011$ ). Necrophilous insects were not using the sticky traps as visual cues.

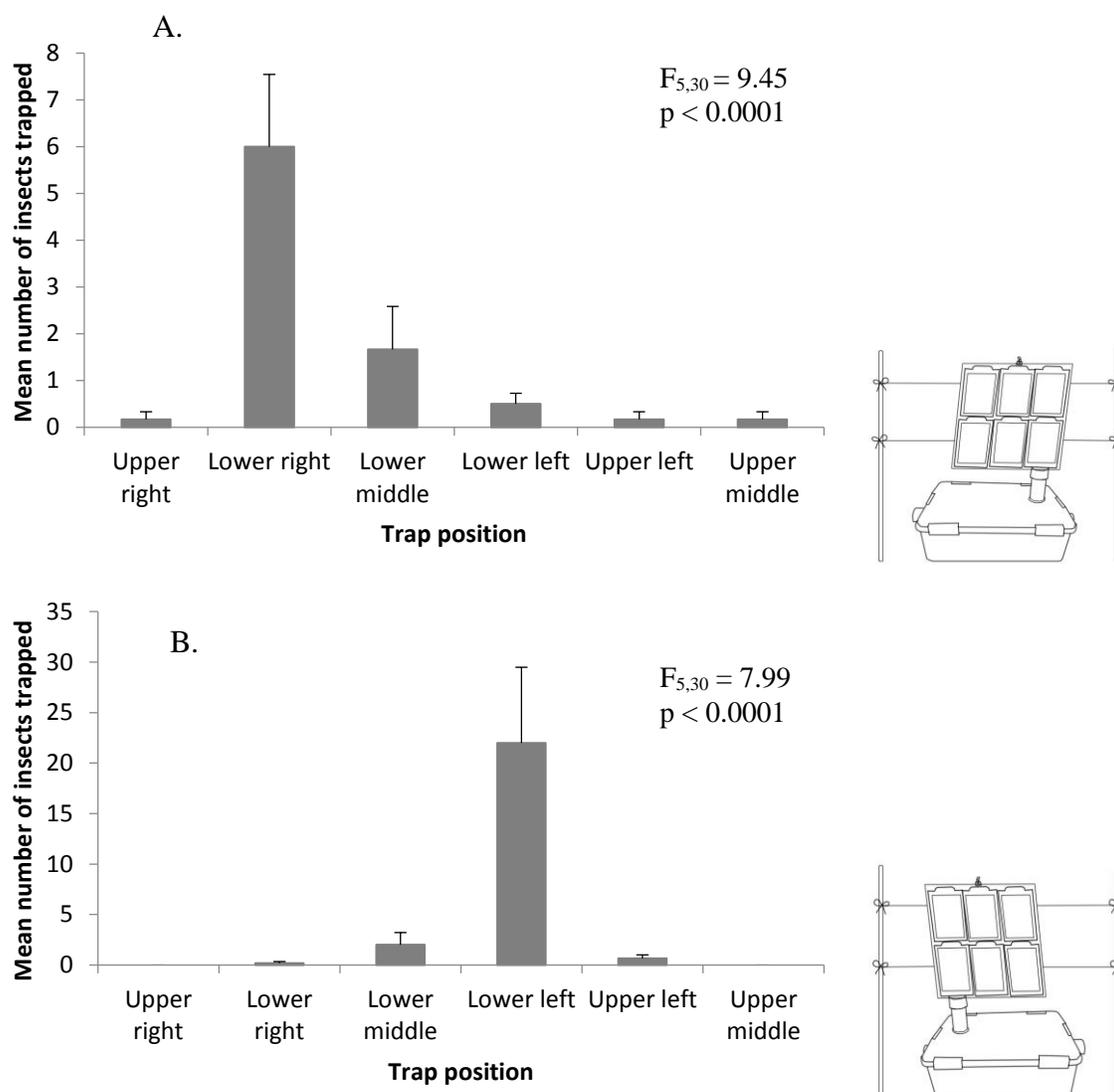


Figure 4. Effect of trap size and orientation on mean number of insects ( $\pm$  SEM) trapped in each trap position facing West (A.) and East (B.). The schematics illustrate both sides of the large trap. Six traps were glued to each of two 41 x 41 cm pieces of poster board and attached by a binder clip to an equal sized piece of corrugated plastic. Mason line was threaded through the corrugation and attached to two pieces of rebar. Significantly more insects were trapped on the downwind East side of the trap (note different Y-axis scales; two-sided paired

t-test,  $T = 2.44$ ,  $df = 5$ ,  $p = 0.03$ ) and on the trap position just above the chimney (one-way ANOVA followed by Tukey's HSD test; A.  $F_{5,30} = 9.45$ ,  $p < 0.0001$ ; B.  $F_{5,30} = 7.99$ ,  $p < 0.0001$ ).

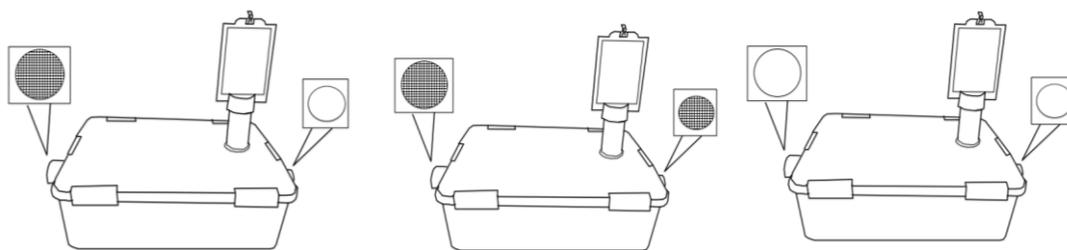
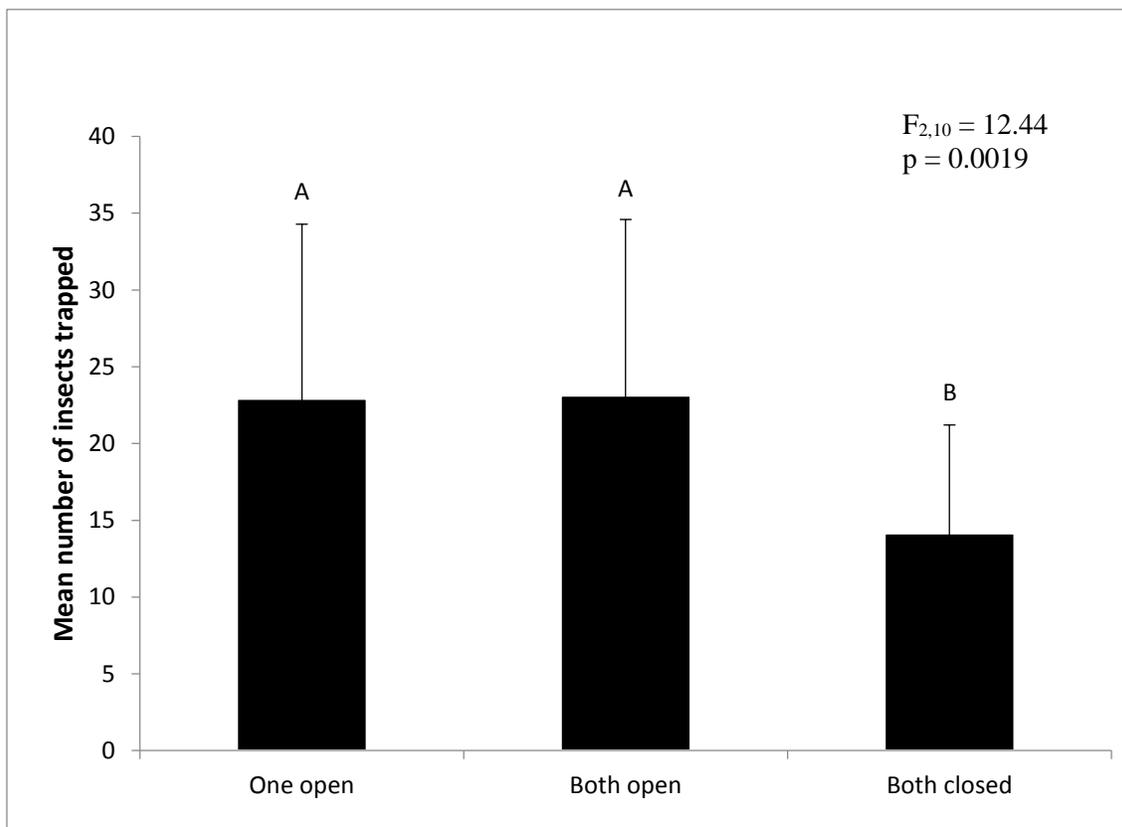


Figure 5. Effect of side openings on the number of insects trapped by the “vented-chamber.”

The leftmost treatment (one side open) served as the positive control. Open sides were covered with window screening to prevent insect entry, and closed sides were capped with 5

cm PVC caps. Bars show mean values  $\pm$  SEM, and bars labeled with the same letter are not significantly different (glmm:  $F_{2,10} = 12.44$ ,  $p = 0.0019$ ; Tukey's HSD test,  $p < 0.05$ ).

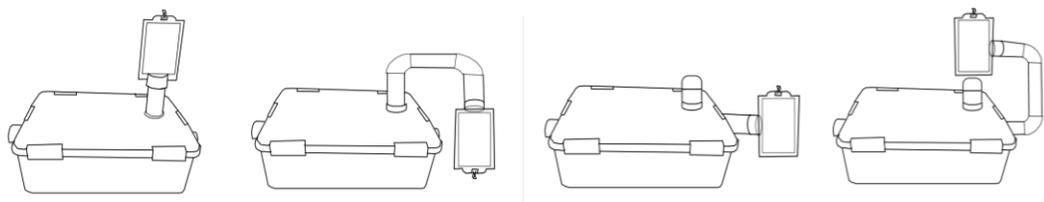
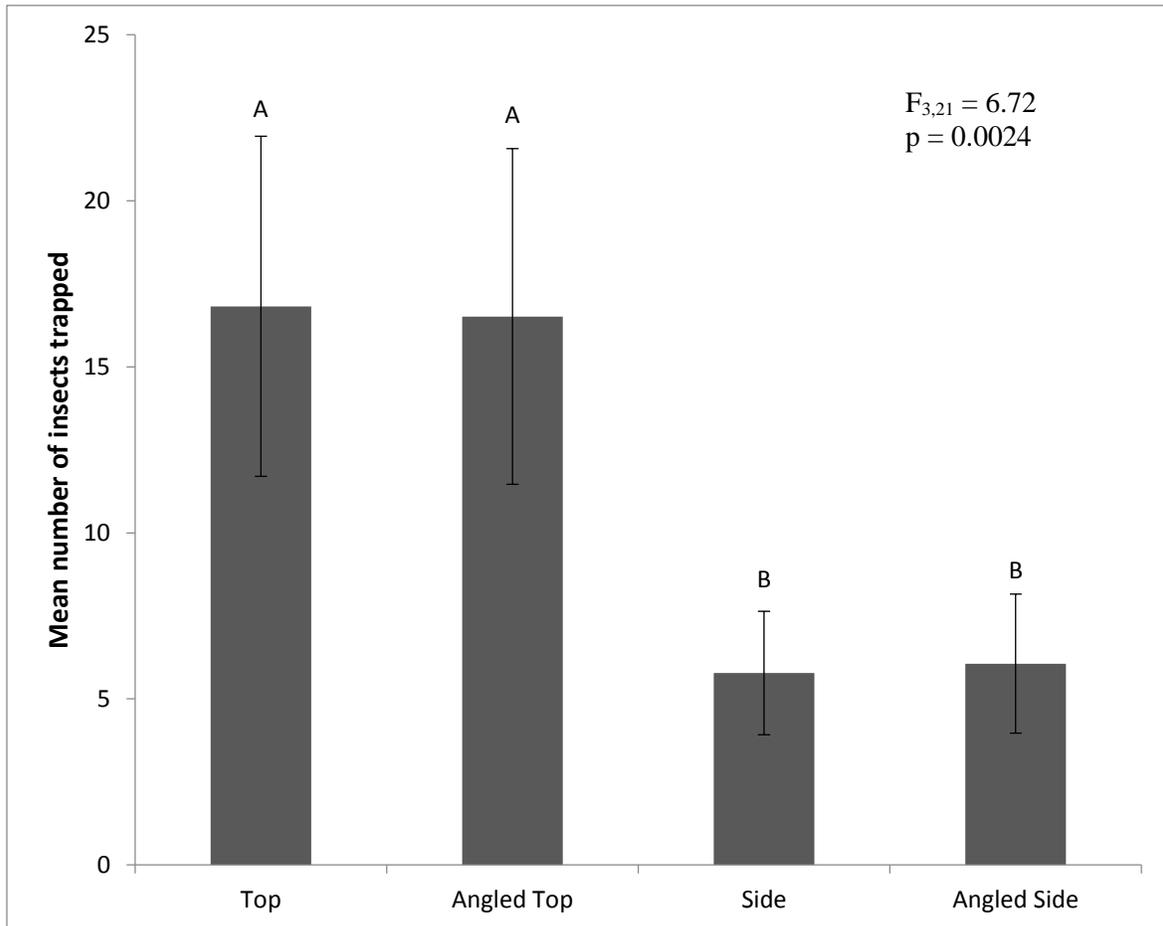


Figure 6. Effect of chimney orientation on the number of insects trapped by the “vented-chamber.” For all treatments, the port on the left side of the chamber was open and covered with window screening. Closed ports were capped with 5 cm PVC caps. The leftmost treatment (top) served as the positive control. Bars show mean values  $\pm$  SEM, and bars

labeled with the same letter are not significantly different (glmm:  $F_{3,21} = 6.72$ ,  $p = 0.0024$ ; Tukey's HSD test  $p < 0.05$ ).

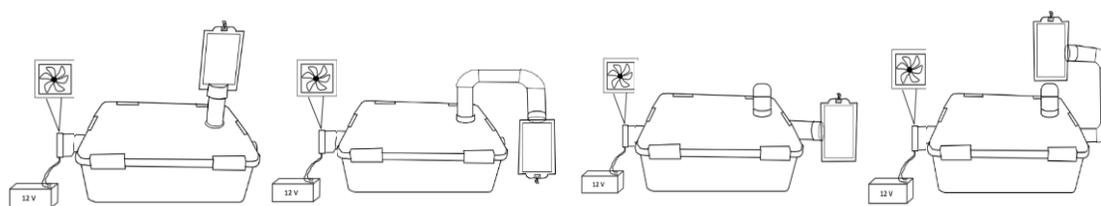
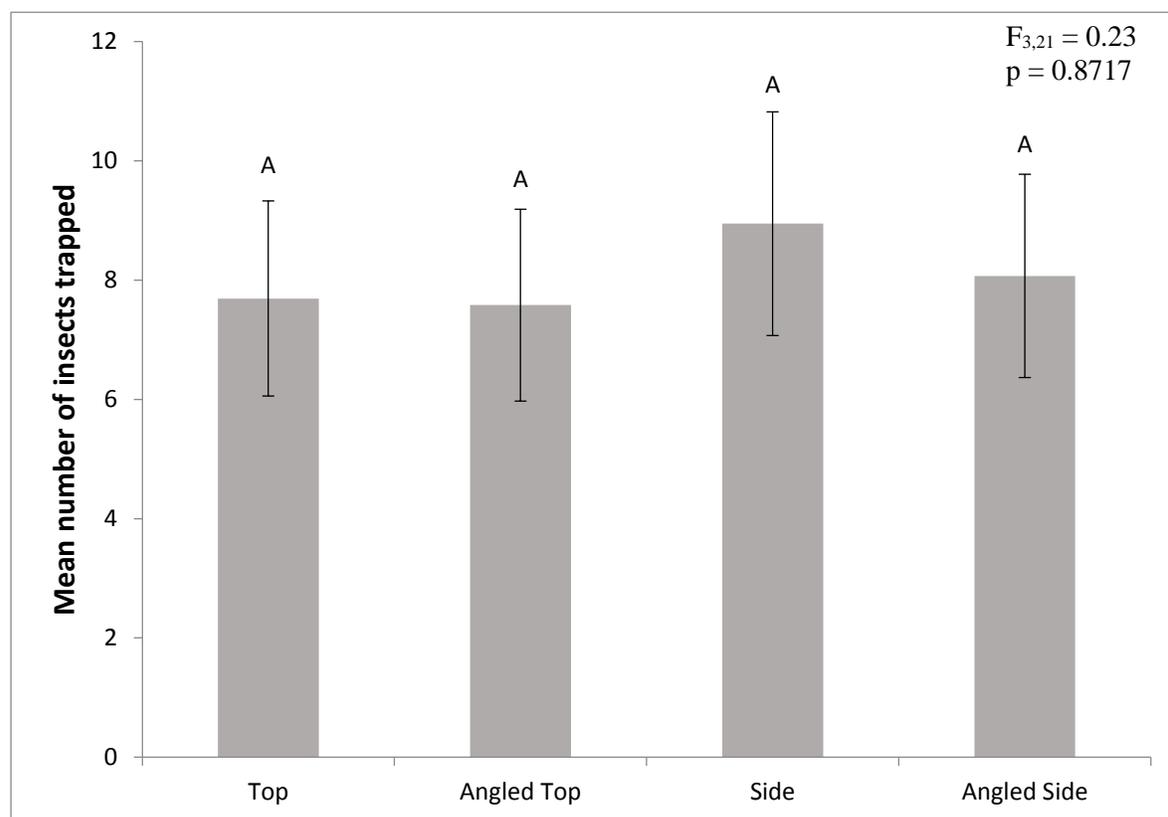


Figure 7. Effect of increasing airflow on the number of insects trapped by the “vented-chamber” with different chimney orientations. A computer fan powered by a 12V battery pushed ambient air through the port on the left side of the chamber, which was covered with window screening. Closed ports were capped with 5 cm PVC caps. Bars show mean values  $\pm$  SEM, and bars labeled with the same letter are not significantly different (glmm:  $F_{3,21} = 0.23$ ,  $p = 0.8717$ ; Tukey’s HSD test,  $p < 0.05$ ).

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**CHAPTER 4.**

**Ecological succession of adult necrophilous insects on neonate *Sus scrofa domesticus* in  
central North Carolina**

**Abstract**

1. The necrophilous insect fauna on carcasses varies seasonally and geographically. Because ecological succession is critical to calculating a postmortem interval (PMI), its documentation assists forensic entomologists in both research and practice.
2. The ecological succession of insects arriving to neonate pig carcasses in central North Carolina during late summer was sampled using a novel “vented-chamber” collection method.
3. Six blow fly species were collected, as well as flesh flies and house flies. Ten beetle taxa were collected, including four species of scarab beetles. Although scarabs are not commonly used in PMI estimations, and are thus not typically reported in forensic literature, they were consistently found on pigs in our experiments.
4. Necrophilous fly activity dominated the first several days of decomposition. Beetle numbers remained low until day 4. By day 7, more than 50% of the pig carcasses were skeletonized and attracting few ( $1.0 \pm 0.76$ ) insects.
5. Necrophilous insects were classified ecologically as carrion-associated, insect-associated (predatory or cannibalistic), or dung-associated. These classifications were useful in explaining their observed arrival patterns.
6. A standardized approach to documenting ecological succession is presented. Differences in the taxa and successional patterns documented in our experiment and a previous study in the same location highlight the ecological variation in such

investigations, and underscore the need for standardization, as well as for more microgeographic ecological succession studies.

## **Introduction**

The documentation of ecological succession of the local fauna present on decomposing model organisms is a critical component of forensic entomology research. Necrophilous insects are attracted to and colonize a decomposing body, whether human or carrion, in a predictable sequence [1-5]. In the practice of forensic entomology, this predictable successional pattern along with environmental parameters such as temperature, are used to calculate a postmortem interval (PMI), or time since death. However, the taxa attracted, their development times, and the successional sequence itself vary by host, season, and geographic region; they may even vary on a microgeographic scale, such as between urban and rural environments within the same city [5-9].

Seasonal and geographic variability in insect succession are frequently addressed topics in forensic literature. While certain forensically-significant insect taxa like *Musca domestica* are cosmopolitan, the vast majority of necrophilous insect taxa have more limited distributions. This natural variation in the distribution of taxa obviously influences the successional pattern across locations. Certain invasive necrophilous species, such as the blow fly *Chrysomya rufifacies*, may even influence the typical arrival pattern of native species [10]. It is important to document the succession of local fauna and reevaluate it in light of species invasions or climate-driven changes in species distributions. Seasonal differences

must also be considered, as different species are indicative of different seasons, even in the same locality. For example, *Calliphora vicina* is a cool weather species in central NC, most commonly sampled in the fall and winter and absent in the summer [11].

This research had three primary objectives. First, we aimed to document the succession of necrophilous insects in central North Carolina. Because ecological succession has been described on juvenile pig cadavers in this locality [11], our use of neonate pigs extends these findings to a smaller decomposition model of ecological succession. Second, in response to difficulties collecting large samples from small pigs, we developed a “vented-chamber” method (Figure 1) to document succession. This method has significantly increased the numbers of sampled insects compared to traditional sampling methods like sweep netting (Chapters 2, 3). Moreover, because this trap eliminates all but olfactory cues, this study represents the first time, to our knowledge, that ecological succession is documented based solely on olfaction. Finally, in our preliminary field work, we consistently found beetles in the family Scarabaeidae (AMC personal observations) on carrion, so this study aimed to document the arrival pattern of these dung-associated beetles. These beetles are rarely included in successional documentation, as they are generally considered incidental fauna at a body, rather than primary colonizers and decomposers of forensic significance [12].

## **Materials and Methods**

**Experimental Animals** Stillborn pigs were acquired from North Carolina State University's Swine Educational Unit. A total of 8 pigs (*Sus scrofa domesticus*) were used, each weighing roughly 1.5 kg. Pigs were placed in a freezer immediately after birth and remained fully frozen until they were placed in the field. This prevented early decomposition and ensured that all pigs were the same temperature at the start of the experiment.

**Study site** This experiment was conducted during September 2015 in an open field at North Carolina State University's Lake Wheeler Road Field Lab in Raleigh, NC (35.731424, -78.667759) (Figure 2c). Pigs were positioned in the field by the northern field edge so that they experienced full daytime sun.

**Field Methods** During the afternoon of the first day of the experiment, eight decomposition sites were established 25 m apart in the experimental field (Figure 2c). At each site, soil was shallowly excavated and spread ~ 4 cm deep atop a standard plastic cafeteria tray (35 cm x 45 cm). Eight fully frozen pigs were placed on the 8 soil-covered trays. This arrangement allowed us to move each pig into the "vented-chamber" (described below) during sampling periods. Pigs were photographed each day prior to sampling to document insect activity and the stage of decomposition. Stages of decomposition were as defined by Kreitlow [13]. Photographs and descriptions of decomposition stages are shown in Figure 3. When not sampled, the carcasses were protected from scavengers within cages constructed of poultry netting that allowed for normal insect colonization.

***Sampling procedure*** Pigs were simultaneously sampled three times each day between noon and 5PM, beginning 24 hours after their placement in the field. Insect sampling was achieved through the passive, “vented-chamber” method, which directed thermally convected decomposition odors to a pair of sticky traps, as outlined in Chapter 3 (Figure 1). At each sampling event, each pig on a cafeteria tray was placed in the chamber and an airtight lid placed on top. Back-to-back unscented Super Catchmaster<sup>®</sup> glue traps (Catchmaster, AP&G Co, Inc., Bayonne, New Jersey) were attached to the chimney atop the chamber and allowed to collect insects for 15 minutes. Each pig was sampled three times daily for seven consecutive days, with a 15 minute rest period between sampling intervals. During the rest period, the pig and tray were removed from the chamber and placed on the ground, allowing decomposition and colonization to occur freely.

To sample beetles, hand-collections were performed on the pig both before and after its placement in the “vented-chamber.” Beetles were stored in 70% ethanol.

**Identifications** Orders Diptera and Coleoptera were the main sampling targets, with emphasis on necrophilous families commonly used in forensic entomology for PMI determinations (Table 1) [12]. The local fauna of interest is also reported in Cammack et al. [11]. Because of their significant role as primary colonizers of decomposing bodies, calliphorid blow flies were further identified to species using Whitworth’s taxonomic key [14]. Beetles were identified with assistance from taxonomists in the North Carolina Plant Disease and Insect Clinic, as well as Almeida and Mise’s forensic Coleoptera key [15]. Insects on sticky traps were identified in situ.

***Ecological associations*** Collected insects were further classified by their ecological associations. We defined these associations by three categories: carrion (C), predatory or cannibalistic insects (I), and dung (D) (Table 1). Carrion insects included insects directly associated with carrion for feeding or breeding purposes, and they included all of the blow flies, flesh flies, and necrophagous beetles. Predatory or cannibalistic insects had ecological associations with another insect (hence “I”) in the carrion microhabitat. While many of the taxa opportunistically feed on dung, only insects with a preference for or well-documented association with dung, such as the scarab beetles, were classified as dung insects. Ecological associations were not mutually exclusive, with several insects fitting into more than one category.

***Statistical analysis*** The number of taxa and the number of insects trapped on each of the eight pigs were compared on each day of decomposition with a one-way ANOVA and Tukey’s HSD (SAS 9.4, SAS Institute Inc.). Linear discriminant analysis was conducted in JMP Pro 13.1.0 (SAS Institute Inc.) on percentage representation of each taxon by day of decomposition.

## **Results**

***Decomposition pattern*** Weather conditions were ideal for a succession experiment, with no precipitation and a gradual increase in ambient temperature during the 7 sampling days (Figure 2a). The average ambient temperature during the 7-day experiment was  $20.65 \pm 0.88^{\circ}\text{C}$  (SEM). Winds were predominantly Northeasterly and Southeasterly throughout the

experiment (Figure 2b). We minimized between-pig variation by using pigs of the same size, body temperature, and level of concealment at the field edge (Figure 2c).

Over half of the pigs (62.5%) fully progressed through decomposition (fresh to skeletal stages) over the 7 days of the experiment (Figure 4). Pigs were in the fresh stage for the longest duration (Figure 4), likely due to the frozen state of the pigs at the start of the experiment and cooler average temperatures during the first half of the experiment (Figure 2a); rate of decomposition is well correlated with ambient temperature [16, 17].

***Richness, overall abundance, and succession*** In total, we trapped and hand-collected 8 necrophilous fly taxa and 10 necrophilous beetle taxa, including four species of scarab beetles (Table 1). The average number of insect taxa varied by day but followed a general bell-shaped curve (Figure 5a).

The number of necrophilous insects also followed a bell curve (Figure 5b). The relative abundance of dipterans and coleopterans varied with the decomposition stages of the pigs. Flies were most abundant, and they were most represented during the early stages (Figure 6). Necrophilous beetles of many species, however, arrived during and after the later decay stage, which started for most pigs on day 4 (Figures 4, 6).

By day 7, the number of insects trapped per pig averaged only  $1.0 \pm 0.76$  insects (Figure 5b). At this point, 5 out of 8 (62.5%) pigs were in the skeletal stage of decomposition (Figure 4).

Linear discriminant analysis of the relative abundance of each taxon by day showed that only the first two canonical relations had high eigenvalues and were significant

(Canonical 1,  $p < 0.0001$ ; Canonical 2,  $p = 0.0086$ ). Linear discriminant analysis indicated significant differences among days of decomposition (Wilk's lambda = 0.0024,  $F = 3.0866$ ,  $p < 0.0001$ ). Days 1 and 2 however did not separate significantly, as indicated by overlap of their 95% confidence ellipses. Day 3 and day 4 separated clearly from each other and from all other days, whereas days 5–7 broadly overlapped (Figure 7). The rays in the biplot (not shown), representing the loading of taxa relative to the position of pig age groups, indicated that *Phormia regina*, *Lucilia sericata* and *Lucilia cuprina* associated (loaded) toward days 2–3, whereas *Cochliomyia macellaria* loaded towards day 4. The beetles generally loaded toward days 5–7.

***Diptera succession*** Table 2 details individual and total dipteran taxa abundance across all experimental days as well as the proportion of pigs each day from which each taxon was trapped on sticky traps. In total, we collected 3,299 flies on all 8 pigs over 7 days. As expected, Diptera was the first major order of insects detected on the pig carcasses. Blow flies and flesh flies were present on pigs as early as day 1 (Table 2). For the first three days of decomposition, flies were trapped from all 8 pigs, and the total dipteran abundance steadily increased between days 1 and 3 (Table 2, Figure 8).

Dipteran abundance peaked on day 3, corresponding with the bloat stage for most pigs (Figure 8). Both the abundance and relative proportion of pigs with adult flies continually decreased after this (Table 2, Figure 8).

Calliphorids were the most abundant group of flies found on the pigs (Table 2). All 6 species of calliphorids were trapped on pigs beginning on day 1. These species were sampled

from every pig on day 2 and/or day 3 (Table 2). Of the blow flies, *Lucilia coeruleiviridis* and *Lucilia illustris* were most abundant on days 1–2 and decreased in relative abundance over the next 4 days (Table 2, Figure 8). Conversely, *Phormia regina*, *Cochliomyia macellaria*, *Lucilia sericata* and *Lucilia cuprina* were less represented on day 1 and increased in relative abundance from days 4–7 (Table 2, Figure 8). The relative abundance of *P. regina* steadily increased from days 1–6, and it and *L. sericata* were the only calliphorid flies trapped on pigs on day 7 of decomposition, but in extremely low numbers (Figure 8).

Sarcophagids, while present on pigs during all seven days of decomposition (Table 2, Figure 8), never reached high overall abundance compared to calliphorids (Figure 8). Unlike the blow flies, sarcophagids were never sampled from all pigs in the same day (Table 2).

House flies, *Musca domestica*, only appeared on pigs from days 3–6 (Figure 8) and were low in abundance throughout this entire interval (Table 2). Their abundance and proportion of pigs from which they were trapped peaked on day 4 (Table 2).

***Coleoptera succession*** Beetle activity began on day 2, a day later than the first fly arrival, and progressed through day 7 (Table 3, Figures 6, 9). Beetle numbers on days 2 and 3 were low (<10), but increased more than 10-fold between days 3 and 4 (Table 3). This coincided with a shift to the decay stage of pig decomposition (Figure 4). On all days except day 7, however, fly abundance was greater than beetle abundance (Figure 6).

The successional pattern of beetles progressed in accordance with their ecological roles. Beetles that were categorized as both carrion-feeders and dipteran predators, including the silphid beetle *Necrophila americana*, the histerid beetles, and the staphylinid taxa, arrived

after maggots were present on the body but before the carcass tissue was depleted (Tables 1, 3). The arrival of these taxa occurred on either day 3 (histerid beetles) or day 4 (all others), when the pigs were either bloated or in the decay stage of decomposition (Figure 4). During these stages, the beetles could feed freely on either maggots or pig tissue.

Three of the four scarab taxa (*Onthophagus pennsylvanicus*, *Onthophagus taurus*, and *Phanaeus vindex*) did not arrive until day 4 (Table 3, Figure 9). These three taxa were present on pigs for only one or two days, likely due to the depletion of their preferred fecal resource. The remaining scarab, *Onthophagus hecate*, was collected in low numbers on pigs during 5 of the 7 days of the experiment (Table 3, Figure 9).

Dermestid beetles were the latest arriving beetles, with their first arrival to the body on day 5 (Table 3). Most pigs were in the postdecay/dry stage at this time (Figure 4). Dermestid beetles were only found on days 5 and 6, likely because only hair and bone was left on most pigs after this point (Figure 3). Very few dermestid beetles (< 5) were sampled overall (Table 3, Figure 9).

## **Discussion**

From the outset, it is important to stress that our passive trap sampled necrophilous insects as they arrived at the carcass, before they directly interacted with the carcass, hence their attraction to this trap was solely based on olfactory cues. Therefore, the ecological succession we describe excluded other sensory modalities (e.g., visual cues), on-carcass interactions (e.g., competition, mating), and close-range decisions by the insects (e.g.,

whether to oviposit), because the arriving insects could use only volatile olfactory cues emanating from the chimney of the “vented-chamber. All the same, olfactory attraction alone was sufficient to create a comprehensive representation of succession that we could relate to clear taxonomic and ecological succession and to the successive decomposition stages of the pigs. These conserved successional patterns highlight the critical importance of olfaction in ecological succession of necrophilous insects.

The changing overall abundance of various taxa on the 8 decomposing neonate pigs over the 7 day experiment is depicted in Figure 10. Not surprisingly, we found that ecological roles helped to explain the general arrival sequence and relative abundance of insect taxa over time. Insects that directly used the carrion’s tissues for feeding or reproduction (Calliphoridae, Sarcophagidae) arrived first, during the fresh stage of decomposition, as expected [2, 18]. Adults oviposit (Calliphoridae) or larviposit (Sarcophagidae) on the body, and their young feed directly on animal tissues throughout development [3]. Competition between blow flies and flesh flies drives their relative population sizes on a carcass; generally, sarcophagid populations are limited by calliphorid population size, and larviposition by sarcophagids ensures that their larvae get an early start on tissue consumption before becoming outnumbered by calliphorids [19].

Carrion-associated fly activity increased through the bloat stage, as did the buildup of decomposition odors [16, 20, 21]. We observed the largest numbers of blow flies and flesh flies, as well as the highest trapping consistency across pigs for these taxa during the days when pigs were bloated (Table 2, Figure 4), consistent with previous work that evaluated

succession on fully exposed carcasses [3, 13]. Blow fly and flesh fly activity declined after this point, likely due to dwindling resources. After the bloat stage, the feeding activity of large maggot masses begins to open the animal's body, exposing the internal organs [3]. Tissue is then rapidly reduced as the maggots feed and grow [3].

Pivotal to our trap design was the hypothesis that necrophilous insects could assess resource suitability based on olfactory cues. Female blow flies are known to make this assessment prior to oviposition [22], and it follows that the number of adult flies arriving to oviposit and larviposit would decline as existing larvae consume the animal's tissues and thus reduce the amount available for subsequent cohorts. While several fly species were attracted and trapped in small numbers on days 6 and 7, these insects would likely not oviposit or larviposit at this late stage in decomposition. Archer and Elgar observed that most flies sampled after the decay stage of decomposition were non-gravid females searching for a protein source [23].

As blow fly and flesh fly activity decreased after the bloat stage, the activity of dung-associated and predatory/cannibalistic insects increased. The decay stage is known to favor necrophilous beetles that prey on abundantly available fly larvae and use the exposed carrion feces as a resource [3]. It was reassuring to find that these beetles also responded to stage-specific community and decomposition odors. With internal organs exposed in the decay stage, carrion insects had easy access to feces previously enclosed within the intestines. *M. domestica*, which is preferentially attracted to feces over carrion itself, as well as all scarab

beetles were trapped and hand-collected in their highest numbers during this time (Tables 2 and 3, Figures 8 and 9) [12].

Insect activity was scarce during the skeletal stage. This stage is characterized by little insect activity, with mites instead being the main organisms associated with the remaining bones and hair [3, 13, 24]. Mite activity is rarely used in PMI determinations, and research on the subject is sparse [5]. Because we sampled mainly with sticky traps off the carcass, mites were excluded from this succession study.

Hand-collections recovered several taxa that were not readily sampled with the “vented-chamber” trap. The scarab *Onthophagus hecate* was hand-collected on days 2–5 and then again on day 7 (Figures 9, 10). This beetle like other paracoprid species is known to tunnel below its fecal resource, which may explain why it was sampled even after aboveground fecal matter from carrion was depleted [25, 26]. Dermestid beetles, though carrion-associated, were not hand-collected until day 5 (Figures 9, 10), coinciding with the postdecay/dry stage for most pigs (Figure 4). These beetles feed on dry tissue, which is present on carrion during this stage of decomposition [12, 13, 27]. Few dermestid beetles (< 5) were collected overall, possibly because of their preference for hiding in small cavities within the hide [12].

While we agree with Payne et al.’s assertion that viewing decomposition stages as discrete events focuses more on physical changes in the carcass than the actual successional pattern of insects [28], we also found that the standard five stage model of decomposition was useful in relating the ecology of the arriving insects to the physical dynamics of the pig.

In many studies, stage boundaries are not synchronized with major faunal shifts [28]. We suspect that the rapid rate of decomposition in our study, likely due to carcass size and warm, stable, precipitation-free environmental conditions and afternoon-only samplings, may have resulted in the appearance of a more synchronized timing of insect arrivals and more clearly defined stage boundaries. Maximum abundance of different taxa was better correlated with stage boundaries than was arrival time. Decomposition stage does not predict which individual taxa are present on carrion, but it did, in our study, explain the general pattern of arrival for insects fulfilling various ecological roles.

A significant motivation of this study was also a previous study [11] that was conducted at the same location, but used larger pigs, different sampling methods, and various degrees of concealment of the carcasses. Although Cammack et al. [11] did not report quantitative results for each taxon, the black blow fly, *Phormia regina*, appeared to be the most commonly sampled species, as in our study (Table 2). Other flies that were trapped or hand-collected in both experiments included *Cochliomyia macellaria*, *Musca domestica*, and all four *Lucilia* species. *Chrysomya megacephala* and three *Calliphora* species were trapped by Cammack et al. [11] but not in our study. While Cammack et al. trapped *C. megacephala* in spring, summer, and fall, it was not trapped on sun-exposed carcasses. Also, none of the *Calliphora* species were collected in the summer [11], consistent with their cool weather preference and explaining their absence from our pigs which were placed in full sun in late summer.

Of the coleopteran taxa, *Necrophila americana*, unidentified histerid beetles, *Creophilus maxillosus*, other unidentified staphylinids, and *Dermestes* species were found in both studies. We also documented several dung beetle species (*Onthophagus* and *Phanaeus*), but it is unknown whether these scarabs were sampled by Cammack et al.

Several factors may explain the observed differences between the two studies: pig size, sampling time, sampling method, and environmental conditions (level of exposure, landscape). Cammack et al. used 10.2 kg juvenile pigs [11], several kilograms larger than our neonate (1.5 kg) pig models. Although larger pigs generally attract more flies than smaller pigs, and their decomposition rate may be slower, carcass size generally has little effect on insect succession, as long as the same type of animal model is used [29, 30]. The general similarity of our findings to those of Cammack et al. [11], and the similarity of both studies to previous investigations with human, swine, canine, and rat remains suggest that the size of the pig carcasses did not contribute substantially to the slight differences between our study and Cammack et al.

Sampling time also differed between the two studies. Cammack et al. sampled 2 hours after morning civil twilight and 2 hours before evening civil twilight [11], whereas we sampled between noon and 6 PM (6 PM is within the two hours before evening civil twilight). Interestingly, *C. megacephala*, collected by Cammack et al. but not in our study, is one of few blow flies known to oviposit at night [31]. Since Cammack et al. did not distinguish between larval and adult samples, it is possible that *C. megacephala* represented larvae that had developed from eggs oviposited overnight. Moreover, *C. megacephala* was

not found on carcasses in a sun-exposed open field location [11] similar to our placement of the pigs.

We suspect that different sampling methods and environmental conditions contributed most to the successional differences in the same location. We coupled the “vented-chamber” approach, primarily to trap flies, with hand-collections of beetles, whereas Cammack et al. [11] used a modified vacuum and sweep net for adults and hand-collection for larvae. While a modified vacuum (different from Cammack et al.’s) was significantly more effective than sweep netting during peak decomposition (Chapter 2), comparisons across separate studies suggest that the “vented-chamber” was more effective than either of these approaches (Chapter 3). We emphasize again two important points: (1) the “vented-chamber” method samples only newly arriving adults and not adults that are resting on the carcass or emerging from the carcass; and (2) although *C. megacephala* was found to be an indicator species for the summer season in this location, it was not found in exposed sunny locations [11], and we did not find it in any of our sampling. The level of exposure (i.e. sun exposure, amount of tree cover) is known to affect faunal differences [1, 32].

Our quantitative analysis of succession on neonate pigs should provide a resource for local forensic entomology investigations, as well as a framework for an experimental approach for documenting ecological succession on carrion. Several aspects of this work are noteworthy: First, the use of neonate pigs, which are readily available, facilitated not only replication of this work, but also the ease of moving and manipulating carcasses. Second, standardization with the “vented-chamber” as a passive sampling method removed human

bias and the inherent disturbance caused by active sampling with a sweep net or vacuum device. Finally, the high trapping efficiency of the “vented-chamber” makes it a useful model toward standardizing sampling so that successional patterns can be compared across geographic regions, seasons, and carrion types (see [28, 33]). As discussed previously (Chapter 3), this approach could be modified to increase the probability of trapping beetles which tend to orient to the carcass on the ground.

We especially emphasize the need to report quantitative empirical results of sampling forensically relevant arthropods. Often, data are reduced to community similarity or dissimilarity indices and statistically derived indicator species. While these metrics are obviously useful, data on relative abundance of taxa, peculiarities of common vs. rare species, and the temporal changes in community organization tend to be obscured in the analysis. For example, Cammack et al. [11] reported *Cochliomyia macellaria* and *Phormia regina* as summer indicator species on sun-exposed pig carcasses. Our study in the same location confirmed that these two species respectively represented 8.8% and 41.3% of all the flies. *Lucilia coeruleiviridis* (28.6%) and *Lucilia illustris* (9.8%), a known summer active species, were also highly represented in our September samples, possibly because of the summer-fall transition. A qualitative comparison of the two studies cannot be made, however, as the relative numbers of sampled adults and larvae were not reported by Cammack et al.

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Table 1. List of forensically significant insects identified across all samples and their ecological associations. If no species listed, taxonomic identifications ended at the family or genus level. Those insects directly utilizing carrion for feeding or breeding purposes are labeled “C,” predatory or cannibalistic insects are labeled “I,” and dung-feeding insects are labeled “D.”

Order	Family	Genus and species	Ecological Association: Carrion (C), Insects (I), Dung (D)
Diptera	Calliphoridae	<i>Lucilia illustris</i> (Meigen)	C [12, 34, 35]
		<i>Lucilia coeruleiviridis</i> (Macquart)	C [12, 35]
		<i>Lucilia sericata</i> (Meigen)	C [12, 34]
		<i>Lucilia cuprina</i> (Wiedemann)	C [12]
		<i>Phormia regina</i> (Meigen)	C [12]
		<i>Cochliomyia macellaria</i> (Fabricius)	C [12, 35]
	Sarcophagidae	Sarcophagidae spp.	C [19]
	Muscidae	<i>Musca domestica</i> (Linnaeus)	D [12]
Coleoptera	Silphidae	<i>Necrophila americana</i> (Linnaeus)	C, I (dipteran predator and cannibal) [12]
	Histeridae	Histeridae spp.	C, I, D (dipteran predator and cannibal) [12, 36]
	Staphylinidae	<i>Creophilus maxillosus</i> (Linnaeus)	C, I (dipteran predator) [12]
		<i>Platydracus</i> spp.	C, I* (*suspected dipteran predator) [12]
		Staphylinidae spp.	C, I (dipteran predator) [5, 24, 37]
	Dermestidae	<i>Dermestes</i> spp.	C, I (cannibalistic) [12, 38]
Scarabaeidae	<i>Onthophagus hecate</i>	D [12, 39]	
	<i>Onthophagus pennsylvanicus</i>	D [12, 39]	
	<i>Onthophagus taurus</i>	D [12, 39]	
	<i>Phanaeus vindex</i>	D [38, 39]	

Table 2. Total abundance of each dipteran taxon across all pigs by day. All dipteran taxa were trapped on the sticky traps of the vented-chamber. In the parentheses following the abundance total is the proportion of pigs (out of eight) in which that taxon was trapped. Boxes that are highlighted in gray indicate when a taxon was trapped on all eight pigs (proportion: 8/8=1). General Diptera abundance and proportion totals are found in the last row of the table, and abundance and proportion totals for each taxon are found in the last column.

Taxon	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Taxon totals
<i>Lucilia illustris</i>	82 (0.875)	114 (1)	103 (1)	22 (0.375)	1 (0.125)	–	–	322 (1)
<i>Lucilia coeruleiviridis</i>	140 (0.75)	411 (1)	284 (0.875)	109 (0.75)	–	–	–	944 (1)
<i>Lucilia sericata</i>	9 (0.375)	21 (0.75)	34 (1)	21 (0.75)	10 (0.5)	–	1 (0.125)	96 (1)
<i>Lucilia cuprina</i>	3 (0.125)	29 (0.75)	40 (1)	8 (0.5)	5 (0.25)	3 (0.125)	–	88 (1)
<i>Phormia regina</i>	32 (0.25)	155 (0.75)	489 (1)	446 (0.875)	161 (0.5)	78 (0.25)	3 (0.125)	1364 (1)
<i>Cochliomyia macellaria</i>	3 (0.375)	6 (0.25)	75 (1)	184 (0.875)	16 (0.25)	6 (0.125)	–	290 (1)
Sarcophagidae	20 (0.5)	67 (0.875)	50 (0.875)	31 (0.75)	4 (0.375)	4 (0.25)	1 (0.125)	177 (1)
<i>Musca domestica</i>	–	–	3 (0.375)	12 (0.875)	2 (0.125)	1 (0.125)	–	18 (0.875)
Daily totals	289 (1)	803 (1)	1078 (1)	833 (0.875)	199 (0.75)	92 (0.5)	5 (0.25)	3299 (1)

Table 3. Total abundance of each coleopteran taxon across all pigs by day. All coleopteran taxa were hand-collected. Those taxa also trapped on the sticky traps of the vented-chamber are denoted with a †. In the parentheses following the abundance total is the proportion of pigs (out of eight) in which that taxon was trapped. Boxes that are highlighted in gray indicate when a taxon was trapped on all eight pigs (proportion: 8/8=1). General Coleoptera abundance and proportion totals are found in the last row of the table, and abundance and proportion totals for each taxon are found in the last column.

Taxon	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Taxon totals
<i>Necrophila americana</i> †	–	–	–	5 (0.25)	3 (0.375)	3 (0.375)	2 (0.125)	13 (0.625)
Histeridae†	–	–	5 (0.25)	42 (1)	22 (0.875)	30 (0.75)	10 (0.625)	109 (1)
<i>Creophilus maxillosus</i>	–	–	–	3 (0.375)	1 (0.125)	–	–	4 (0.5)
<i>Platydracus</i> spp.	–	–	–	1 (0.125)	1 (0.125)	–	–	2 (0.25)
Staphylinidae†	–	–	2 (0.25)	18 (0.875)	42 (1)	24 (0.875)	9 (0.5)	95 (1)
<i>Dermestes</i> spp.	–	–	–	–	1 (0.125)	2 (0.125)	–	3 (0.25)
<i>Onthophagus hecate</i>	–	1 (0.125)	1 (0.125)	1 (0.125)	3 (0.25)	–	1 (0.125)	7 (0.5)
<i>Onthophagus pennsylvanicus</i>	–	–	–	12 (0.625)	5 (0.375)	–	–	17 (0.875)
<i>Onthophagus taurus</i>	–	–	–	2 (0.25)	3 (0.375)	–	–	5 (0.5)
<i>Phanaeus vindex</i>	–	–	–	1 (0.125)	–	–	–	1 (0.125)
Daily totals	–	1 (0.125)	8 (0.5)	85 (1)	81 (1)	59 (1)	22 (0.75)	256 (1)

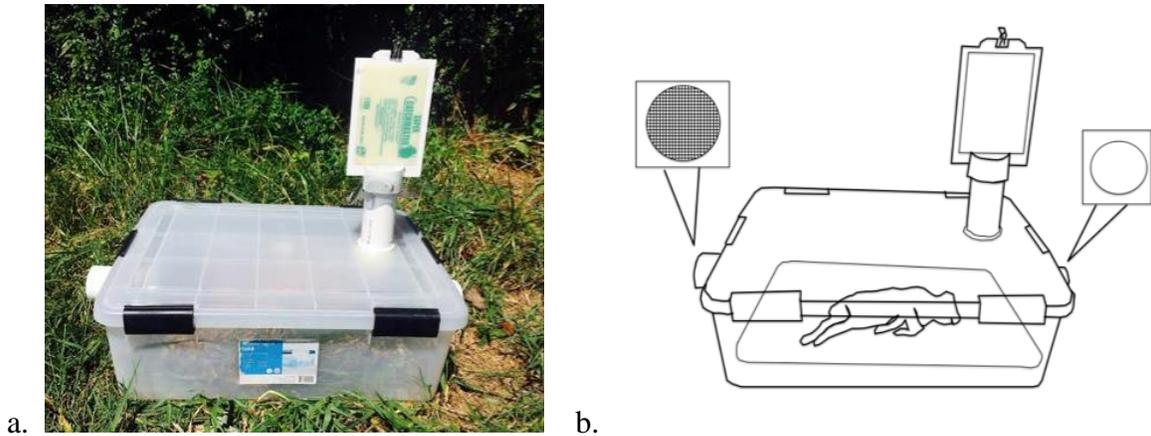
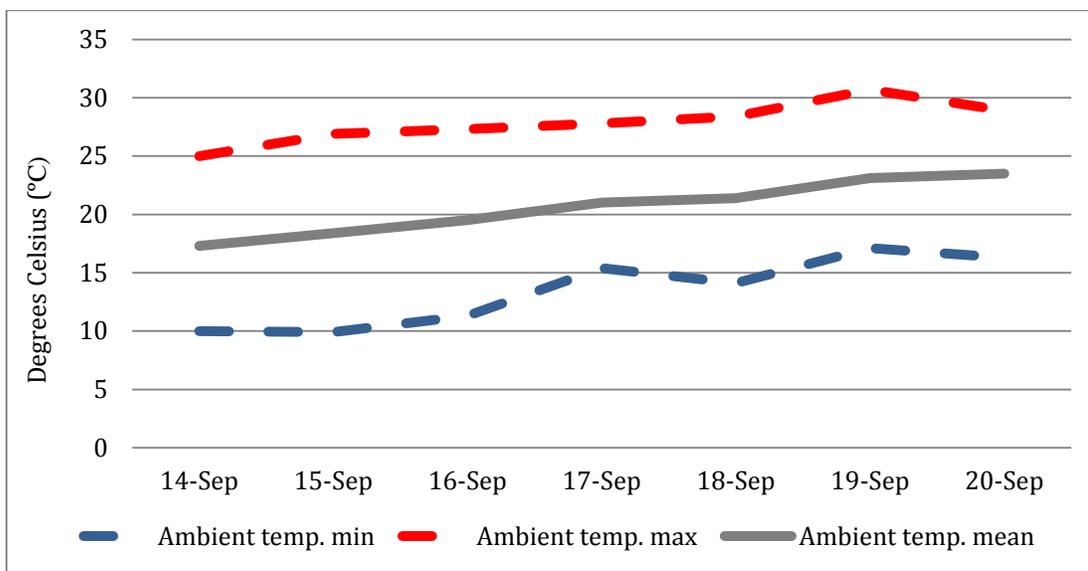
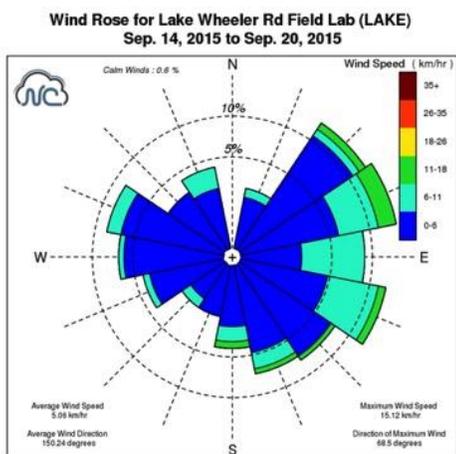


Figure 1. Photograph (a.) and illustration (b.) of the “vented-chamber” method as outlined in Chapter 3. The collection unit consists of a 39 liters airtight chamber with PVC ports. Ports on the left and top of the box, with the orientation as above, were kept open with mesh window screening. Thus, air could flow freely through the chamber, but insects could not enter it. The right port was capped. The top port, or chimney, opened to back-to-back unscented glue traps. Pig orientation in reference to the ports was always as seen above.

Figure 2. Environmental conditions during field experiments. (a) Daily minimum, maximum, and average ambient temperatures ( $^{\circ}\text{C}$ ) near the field research site during the experiment. Temperature data were acquired from the State Climate Office of North Carolina's Lake Wheeler Road Field Lab weather station. There was no measurable precipitation during this time period. (b) The predominant wind directions were from the Northeast and Southeast. Wind data from the State Climate Office of North Carolina Lake Wheeler Rd. Field Station (<http://climate.ncsu.edu/windrose.php?state=NC&station=LAKE>). (c) Experimental field at the Lake Wheeler Road Field Lab in Raleigh, NC (35.731424, -78.667759). All pigs were placed 25 m apart along the dotted line at the northern edge of the field. Pig positioning was kept consistent for all pigs, with the heads facing east. Directionality was conserved between the wind rose (b) and field diagram (c).



a.



b.



c.

Figure 3. Decomposition stages of neonate pigs, showing photographs of pigs at each stage of decomposition and the corresponding characteristics of decomposition stages as defined by Kreitlow [13].



Stage	Photograph	Description
Fresh	A	Begins at death; no apparent physical signs of decomposition; first responders ( <i>Calliphoridae</i> and <i>Sarcophagidae</i> ) arrive
Bloat	B	Abdomen is distended; peak calliphorid and sarcophagid numbers
Decay	C	Abdomen no longer bloated; maggot masses are visible; body open from maggot activity; necrophilous beetles begin to arrive
Postdecay/dry	D	Nothing left on the carcass but skin, cartilage, and bones; stage dominated by beetles
Skeletal	E	Only hair and bones left; few beetles remain, but mites present

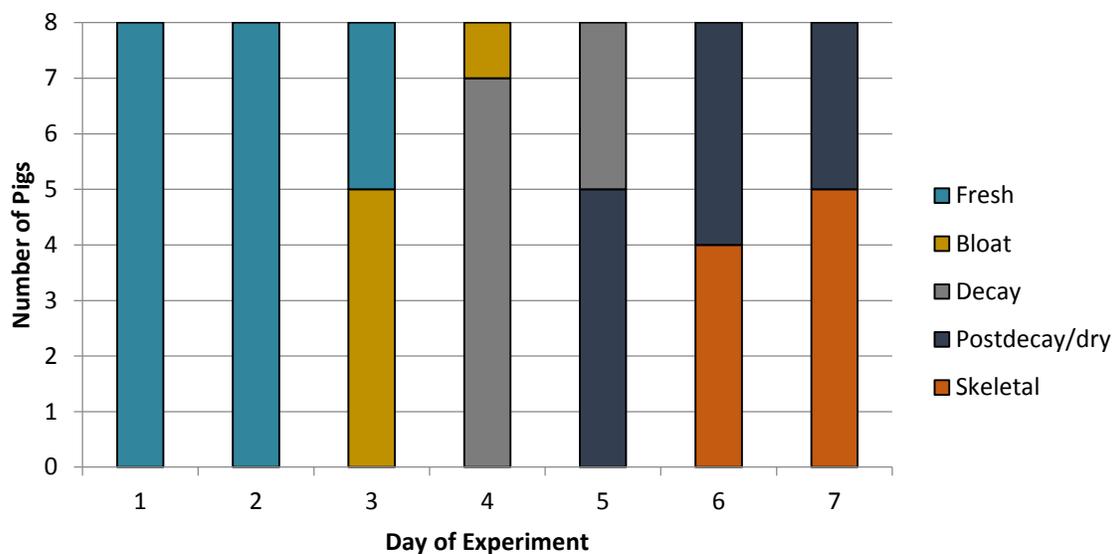


Figure 4. Number of pigs undergoing each of the five stages of decomposition throughout the seven days of the experiment. All pigs ( $n = 8$ ) were of the same weight (1.5 kg) and temperature ( $\leq 0^{\circ}\text{C}$ ) at the start of the experiment. Over half of the pigs were skeletonized by day 7 of the experiment.

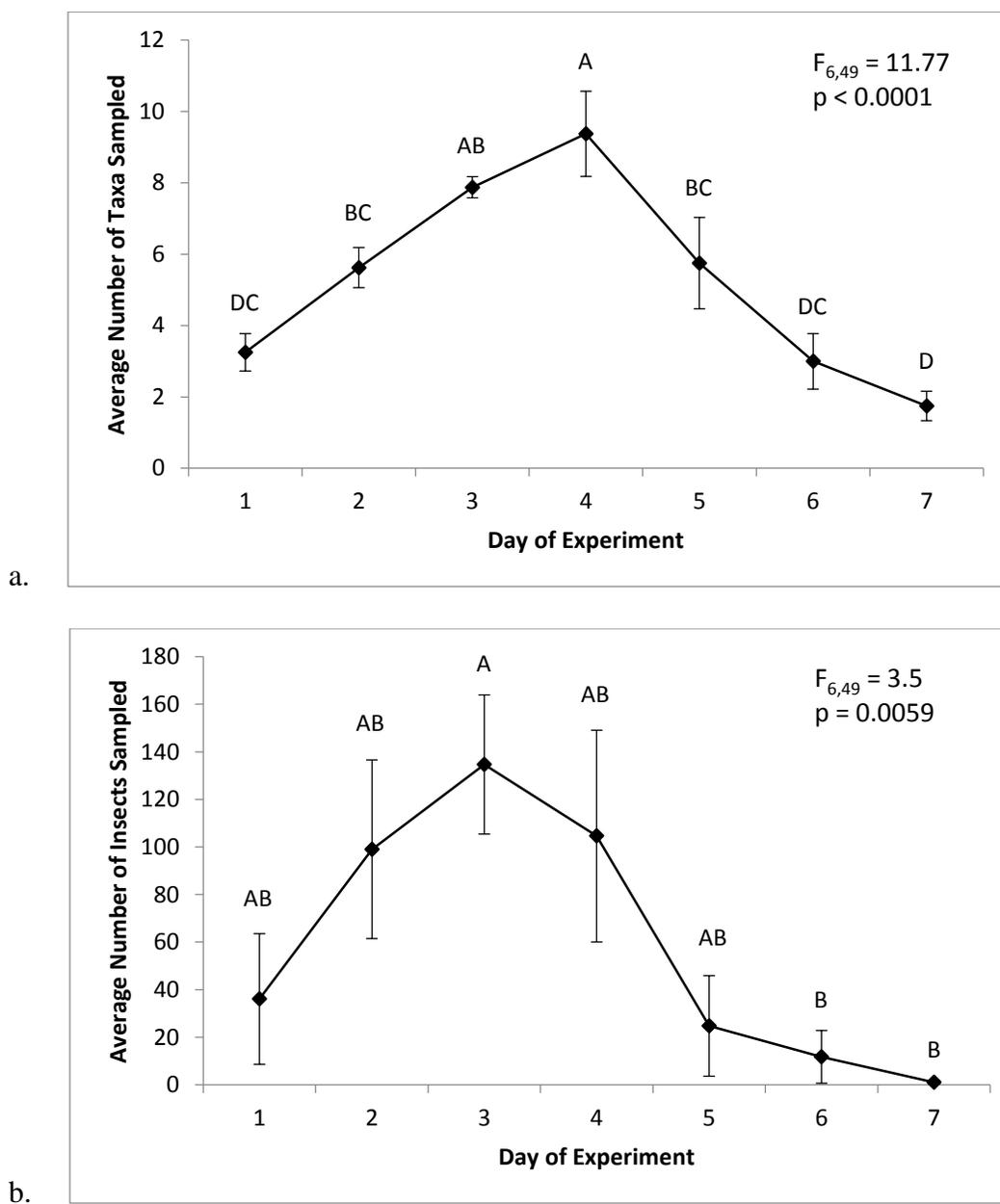


Figure 5. Mean number of (a) taxa and (b) insects sampled from pigs at each day of decomposition. Points and error bars show mean values  $\pm$  SEM. Means labeled with the same letter are not significantly different (ANOVA, Tukey HSD,  $p < 0.05$ ).

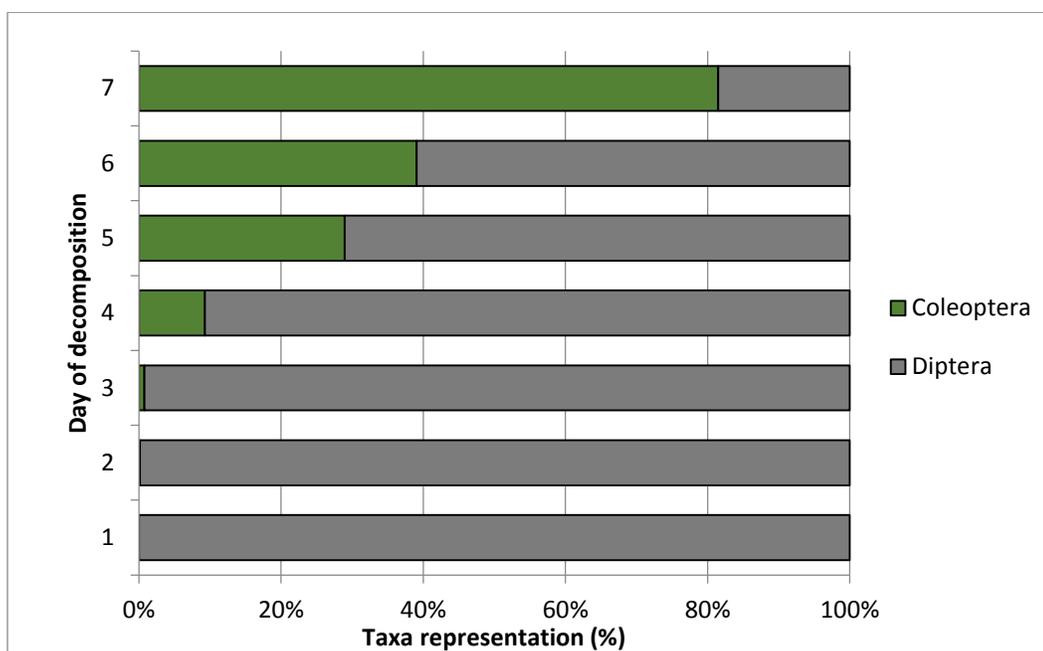


Figure 6. Percentage representation of dipteran and coleopteran taxa trapped or hand-collected from pigs on each day of the 7-day decomposition process. Total insects sampled are shown in Tables 2 and 3.

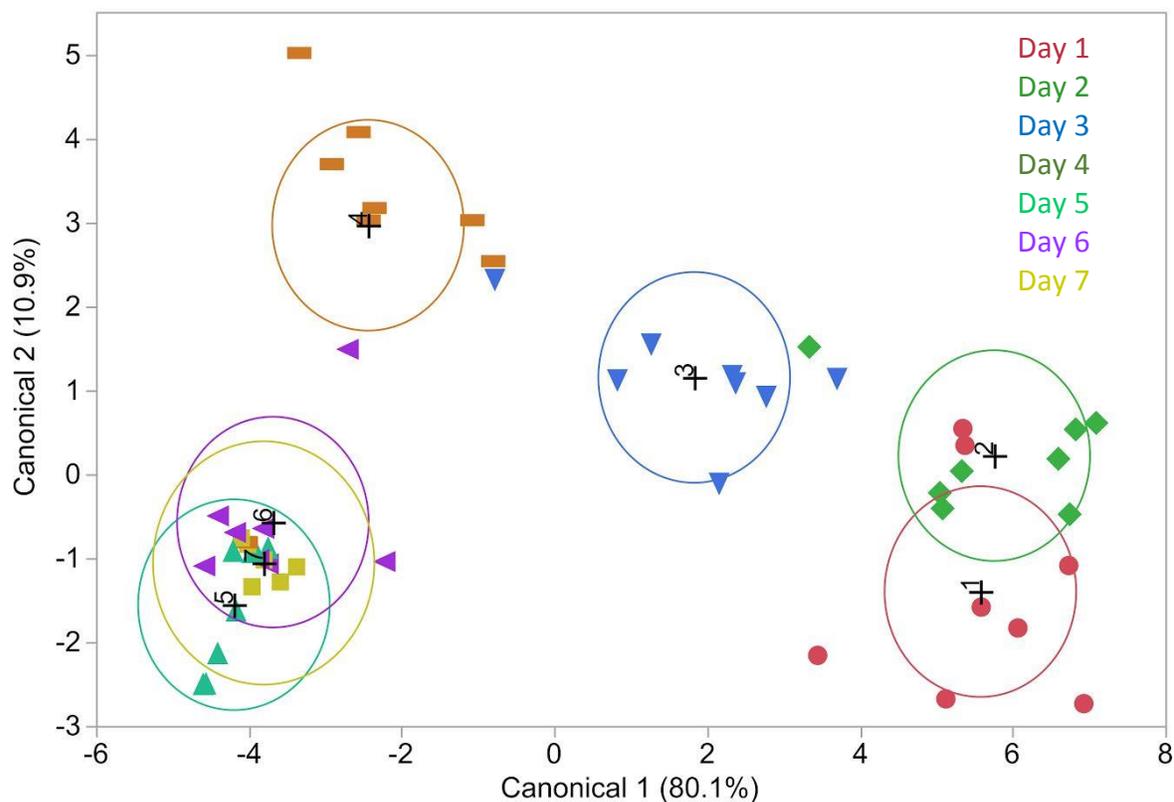


Figure 7. Linear discriminant analysis of 8 neonate pigs over 7 days of decomposition. The percentage representation of dipteran and coleopteran taxa (8 Diptera, 10 Coleoptera) trapped or hand-collected daily was used in the analysis. Color coded numbers represent day of decomposition of each replicate pig. The plus (+) corresponds to each age group mean, with the day in black adjacent to it (rotated 90° counterclockwise), and ellipses represent 95% confidence intervals for each mean.

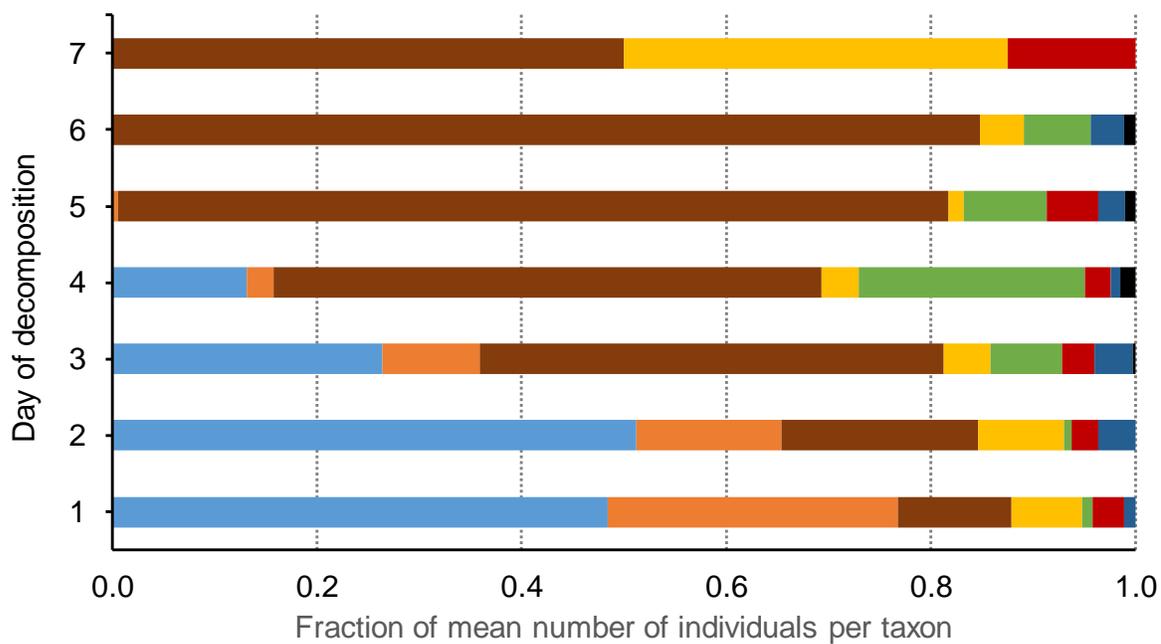
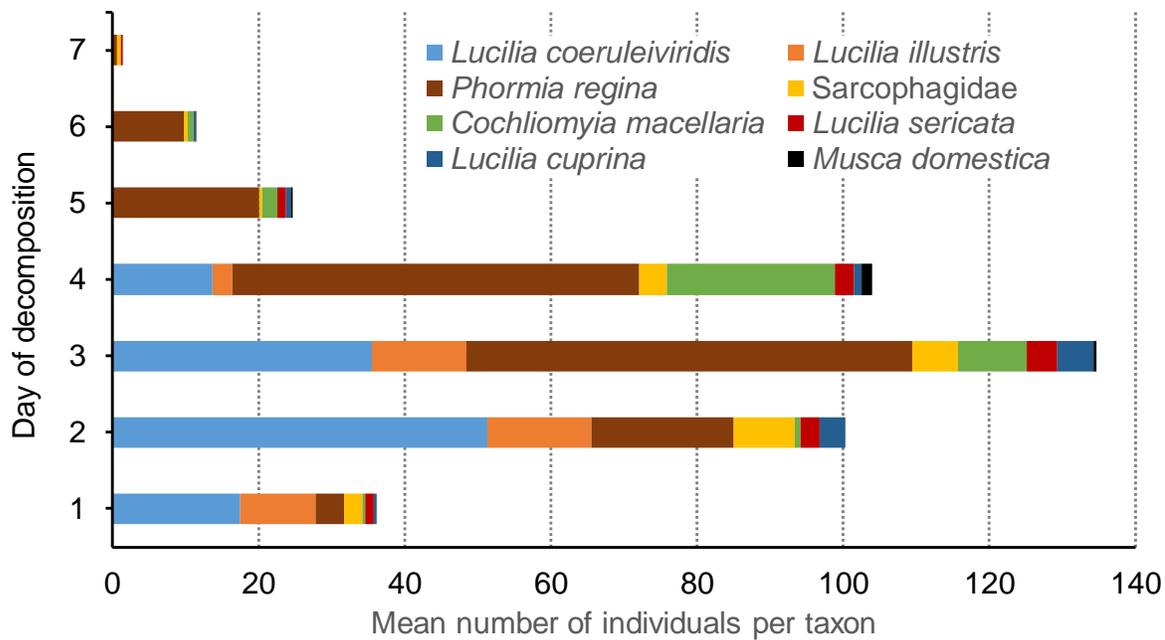


Figure 8. Percentage representation of mean number of dipteran individuals per taxon trapped or hand-collected daily from pigs during the 7-day decomposition process. N = 8 pigs. Total insects sampled are shown in Tables 2 and 3.

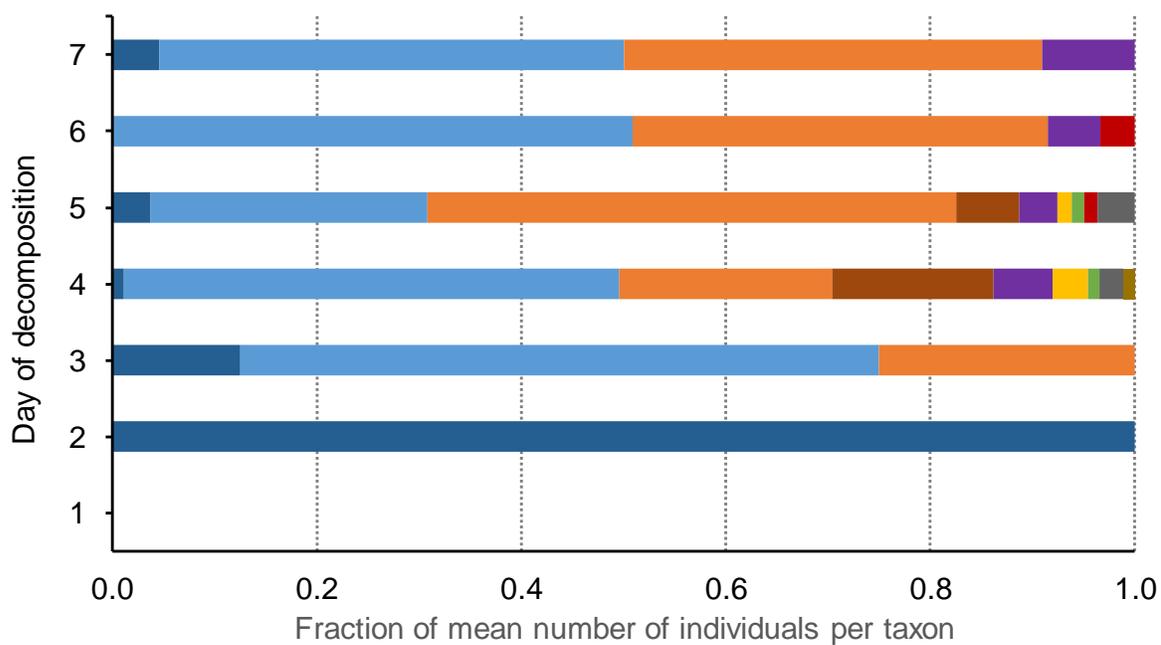
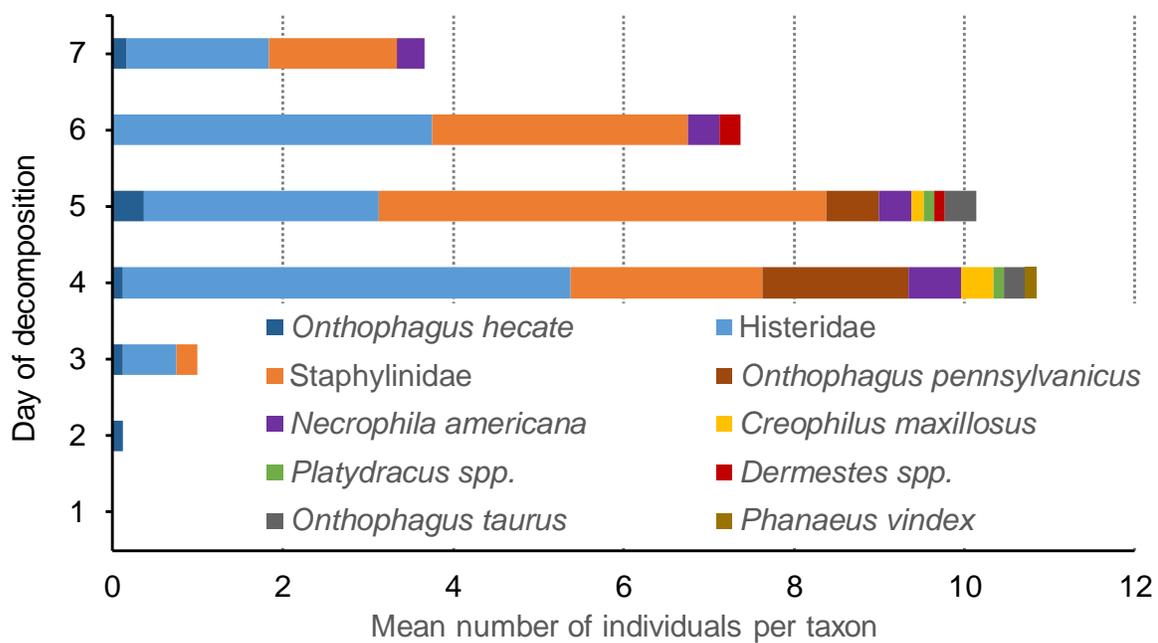


Figure 9. Percentage representation of mean number of coleopteran individuals per taxon trapped or hand-collected daily from pigs during the 7-day decomposition process. N = 8 pigs. Total insects sampled are shown in Tables 2 and 3.

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
<i>L. coeruleiviridis</i>		████████	████████	████████			
<i>L. illustris</i>		████████	████████	████████			
<i>P. regina</i>		████████	████████	████████	████████		
<i>C. macellaria</i>				████████			
Sarcophagidae							
<i>L. sericata</i>							
<i>L. cuprina</i>							
<i>O. hecate</i>							
Histeridae							
<i>M. domestica</i>							
Staphylinidae							
<i>N. americana</i>							
<i>C. maxillosus</i>							
<i>Platydracus</i> spp.							
<i>O. pennsylvanicus</i>							
<i>O. taurus</i>							
<i>P. vindex</i>							
<i>Dermestes</i> spp.							

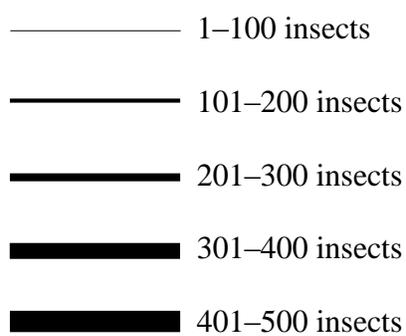


Figure 10. Successional pattern of insects arriving to neonate piglets in central North Carolina in September 2015. Line thickness corresponds to the number of individuals sampled from each taxon daily.

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**CHAPTER 5.**

**Effects of carrion relocation on the succession of necrophilous insects**

**Abstract**

1. As a body decomposes, volatile organic compounds (VOCs) are released into the environment. The odor profile changes over time, as different body tissues are degraded and microbial succession advances.
2. Ecological succession of necrophilous insects on carrion follows a predictable sequence. This predictability is due to differential attraction of various insects to the changing odor profile associated with carrion and its colonizing insects.
3. Nevertheless, the dependency of insect arrival on the duration of the carrion's residency at the site has not been investigated.
4. To assess the fidelity of necrophilous insects to carrion of specific decomposition ages, independent of its location, we monitored the decomposition of neonate pigs in one field and then simultaneously relocated carcasses of different decomposition ages to an ecologically similar but remote field. Arriving insects were sampled with a vented-chamber, which excluded all sensory modalities but olfaction. We examined the effects of decomposition age and relocation on necrophilous insect community assembly.
5. Insect community composition differed over a four-day decomposition period, showing that insects were differentially attracted to pigs of different decomposition ages. Upon relocation to a new field, we found overall concordance between respective decomposition ages in the two fields, with similar relative abundance of similar taxa before and after transfer. Although different decomposition ages

- continued to attract different insects, differentiation of the necrophilous insect communities relative to the age of decomposition was less pronounced after transfer. Several rare taxa were sampled only in field 1 prior to relocation and not in field 2 after relocation.
6. Differential attraction to carrion is odor-driven. Olfactory cues alone were sufficient to differentiate insect communities based on pig decomposition age.
  7. This is the first documentation of the invasive hairy maggot blow fly, *Chrysomya rufifacies*, in central North Carolina. This predatory species poses significant challenges to post mortem interval (PMI) estimations.
  8. The results of this study demonstrate that translocating a decomposing body to a new, but geographically and ecologically similar location continues the predicted insect succession, albeit with greater variance.

## **Introduction**

After death, a series of physical and chemical changes occur within the body as it decomposes. Decomposition begins within minutes of death, as the cessation of essential metabolic functions triggers cellular changes [1-3]. Decomposition first begins with autolysis, a period of cellular self-digestion. While the depletion of oxygen after death destroys cells, anaerobic microbes from the gut and respiratory system thrive in these conditions [2]. The nutrient-rich cell contents released during autolysis fuel the process of

putrefaction, the decomposition of tissue by microorganisms like bacteria, protozoans, and fungi [4].

Aiding and accelerating the process of decomposition are necrophilous insects primarily of orders Diptera and Coleoptera [5-7], without whose feeding activity, the decomposition rate is significantly slowed [8]. Necrophilous insect taxa arrive to a decomposing body in a predictable sequence [9-13]. Several sensory modalities contribute to the predictability of insect succession, but primary among these are vision and differential attraction to volatile organic compounds (VOCs) that are byproducts of decomposition [14-19]. Nearly 80 compounds have been identified in headspace analysis of decomposing pig models [20]. Phenolic molecules (skatole, indole), sulfur- and nitrogen- containing gases (dimethyl di- and tri-sulfides, hydrogen sulfide, ammonia), aliphatic and aromatic hydrocarbons (methane, toluene), esters (butanoic butyl ester, butanoic ethyl ester), ketones (2-nonanone, 2-butanone), alcohols (ethanol, butanol), amino acids (alanine, proline, methionine, GABA), and small carboxylic acids (oxalic acid, propionic acid) are just some of the products produced as carbohydrates, lipids, proteins, and nucleic acids are catabolized in the decomposition process [1, 2, 4, 20-23]. Tissues decompose at different rates, are composed of organic macromolecules in different ratios, and are colonized by different microbial assemblages, so the VOCs that emanate from a body vary over time [21, 24, 25].

Several studies have identified decomposition VOCs that serve as attractant cues for necrophilous insects. Some compounds, such as dimethyl trisulfide, that co-occur in decomposing cadavers [1, 23] [26] and in carrion-mimicking plants like the dead horse arum

[16], attract *Calliphora vicina*, *Lucilia caesar*, *Lucilia sericata* and other calliphorids and even coleopterans, and are considered to be important cues in colonization (of cadavers) and pollination (carrion-mimicking plants) [16, 19].

Many factors are known to influence, delay, or alter the expected successional pattern of insects. Among them are geography [27-30], accessibility/concealment [6, 7, 31, 32], seasonality [9, 33, 34], interspecific competition [35], carrion type [36-38] and level of sun exposure [9, 39]. The time-course of the differential responses of necrophilous insects can alter the successional pattern, yet it is poorly characterized. A central question is: How is succession affected when a body undergoes decomposition in one place and is then transported to another location? In one scenario, insects would respond to specific compounds and blends of compounds that correspond to their preferred decomposition age. Under this mechanism, insects arrive sequentially and respond to a set of compounds independently of what transpired before these compounds were produced. Thus, the successional pattern would be expected to continue unchanged when the body is translocated to a new, ecologically similar site because new responders would be recruited to the carcass. Alternatively, it is possible that persistence of the carcass in one location might serve as a “staging” arena even for late arrivers which might assemble in the area and be primed in the vicinity of the resource in “anticipation” of the proper olfactory cues. If the carcass is relocated to a new site, the assembly of the successional colonizers would then need to start anew.

We sought to elucidate the mechanisms contributing to the predictable succession of necrophilous insects visiting carrion by examining the effect of carrion relocation on insect succession, while excluding all sensory modalities except olfaction. Our approach was to characterize ecological succession on decomposing neonate pigs in one location and then simultaneously move them to a new, ecologically similar location. By using pigs at four different ages within the decomposition process, we were able to assess the differential attraction and community organization of insects to each decomposition age in two locations – where succession commenced in the first location and changes in succession as it continued in the second location. It is important to note that this approach is substantially different from large-scale postmortem transportation of decomposing bodies, such as from an urban to rural area or across geographic regions, where locality-specific differences in insect fauna can assist in criminal cases [6]. Instead, in this study, we assessed post-relocation community assembly within the same microgeographic area, where the same necrophilous taxa are expected. Key to this approach was to exclude all sensory modalities except olfaction, and we accomplished this with a “vented-chamber” sampling method that effectively uncoupled thermally convected odors emanating from a decomposing pig carcass from all other close-range cues.

## **Materials and Methods**

***Experimental Animals*** Stillborn pigs were acquired from North Carolina State University’s Swine Educational Unit. A total of 20 pigs (*Sus scrofa domesticus*), each weighing roughly

1.5 kg, were used in this experiment. Pigs were placed in a freezer immediately after birth and remained fully frozen until they were placed in the field. This prevented early decomposition and ensured that all pigs were the same temperature at the start of the experiment.

**Study site** This experiment was conducted during September and October 2015 in two open fields at North Carolina State University's Lake Wheeler Road Field Lab in Raleigh, NC (Figure 1). One field (35.730605, -78.673048) was designated the decomposition field (i.e., field 1), where pigs were placed and sampled daily. Pigs were positioned in this field at the edge of a tree line with their heads oriented eastward. This ensured that they experienced full daytime sun. The other field (35.731424, -78.667759) was designated the transfer field (i.e., field 2) into which all pigs were transferred and sampled on the fifth day of the experiment, henceforth referred to as the "transfer day" or "day of transfer." Care was taken to select a transfer field that was as similar to the decomposition field as possible in terms of overall sunlight and vegetative characteristics. The two fields were approximately 426 m apart and were separated by two geographical barriers: a stream (waterway) and a dense row of trees. Directionality of pig placement was conserved when pigs were moved to the transfer field.

**Field Methods** Frozen neonate pigs were added sequentially to the decomposition field over a four day period. In September 2015, three pigs were added to the decomposition field each day for 4 days (12 pigs total). At the time of placement, three shallow holes were dug 25 m apart in the decomposition field, and the displaced soil was piled ~4 cm high atop a standard plastic cafeteria tray (35 cm x 45 cm). Each of the three pigs was placed on a soil-covered

tray. This allowed us to move each pig into the “vented-chamber” (described below) during sampling sessions. Pigs were first sampled 24 hours (decomposition age 1) after they had been placed in the field, and then daily for 3 more days. On the fifth day of the experiment, no new pigs were added to the field and the pigs (decomposing for 1–4 days) were sampled in the decomposition field and then relocated to the transfer field. Pigs were sampled in the transfer field beginning 15 min after placement. The experiment was repeated in October 2015, when two rather than three pigs were added each day to the decomposition field (8 pigs total). Thus, in total the combined experiments represented 20 pigs. Scheduling of pig placement, sampling, and transfer is presented in Table 1. When not sampled, the carcasses were protected from scavengers within cages constructed of poultry netting that allowed for normal insect colonization.

***Sampling procedure*** Pigs were simultaneously sampled twice daily between 2 PM and 7 PM, beginning 24 hours after their placement in the decomposition field. Insect sampling was achieved through the passive “vented-chamber” method, which thermally convected decomposition odors to a pair of sticky traps, as outlined in Chapter 3 (Figure 2). At each sampling event, each pig on a cafeteria tray was placed in the chamber and an airtight lid placed on top. Back-to-back unscented glue traps (Super Catchmaster<sup>®</sup>, Catchmaster, AP&G Co, Inc., Bayonne, New Jersey) were attached to the chimney atop the chamber and allowed to collect insects for 15 minutes. Between each sampling interval was a 15 minute rest period. During this time, the pig and tray were removed from the chamber and placed on the

ground, allowing decomposition and colonization to occur freely. Sticky traps were wrapped in cling plastic wrap and stored in the freezer.

To sample beetles, hand-collections were performed on the pig both before and after its placement in the vented-chamber. Beetles were stored in 70% ethanol.

**Transfer** On the transfer day, pigs were sampled for two 15 minute sampling periods in the decomposition field, as previously outlined. At the end of the second sampling period, all pigs were again removed from the vented-chamber and allowed a 15 minute rest period on the ground. Pigs were then enclosed in the chamber and all open ports closed with PVC end caps. This ensured all odors remained inside the chamber, preventing cross-contamination while simultaneously transporting pigs to the transfer field.

Pigs were spaced 25 m apart in the transfer field and removed from the chambers for a 15 minute rest period. Two additional 15 minute sampling periods (vented-chamber and hand-collections) separated by a 15 minute rest period on the ground were then performed.

**Identifications** Collected insects were categorized based on their forensic significance. Orders Diptera and Coleoptera were the main sampling targets, with emphasis on those necrophilous families commonly used in forensic entomology for PMI determinations (Table 2). The local fauna of interest is also reported in Cammack et al. and in Chapter 4 [40]. Because of their significant role as primary colonizers of decomposing bodies, calliphorids (blow flies) were further identified to species level using Whitworth's taxonomic key [41]. Insects on sticky traps were identified in situ.

**Insect community** Linear discriminant analysis was conducted in JMP Pro 13.1.0 (SAS Institute Inc.) on absolute number of each taxon sampled (1) in the decomposition field across days 2–5 of the experiment (decomposition ages 1–4), (2) in the decomposition field on the day of transfer, and (3) in the transfer field after transfer. If no insects were sampled from a pig on a particular day, that data point was excluded from the analysis. Pig decomposition ages were denoted as day 1 – day 4, corresponding to the pig’s time spent in the field (Table 1). For example, pigs placed in the field 24 hours before the transfer day were considered day 1 pigs.

Differences in insect numbers by pig decomposition age both within and between fields were analyzed with one-way ANOVA followed by Tukey’s HSD test.

## **Results**

***Weather conditions*** The average temperature during the September dates of the experiment ( $22.1 \pm 1.1^\circ\text{C}$ ) was more than twice the average temperature during the October dates ( $9.9 \pm 1.1^\circ\text{C}$ ). During the September experimental dates, between 1.3 and 18 mm of rain fell each day, either before or after sampling (State Climate Office of NC). There was no precipitation during the October dates.

***Decomposition*** Pig decomposition progressed at different rates during the two sets of experimental dates (i.e., seasonal differences). Decomposition during October was slower than in September. All but day 1 pigs in the September study had progressed to the point of abdominal opening, whereas no pigs in the October study reached this point. Throughout this

study, we use “decomposition age” to refer to a pig’s age in the field and its daily progression through the decomposition process, rather than to denote discrete physical and insect community markers. Pigs spent several days in each of the discrete decomposition stages, as defined by Kreitlow [42], so defining decomposition ages as daily changes accounted for differences among pigs without necessarily synchronizing them to discrete decomposition stages.

***Succession of the insect community in the decomposition field*** The overall undisturbed ecological succession in the decomposition field is shown in Figure 4. Linear discriminant analysis of the insect community structure across all four sampling days showed significant overlap between day 1 and day 2 pigs, but day 3 and day 4 pigs had significantly different community structures, indicated by the lack of overlap of their 95% confidence ellipses with each other and with any other groupings (Figure 4a). Day 1 and day 2 pigs were relatively tightly grouped, indicating minimal variation in their insect community structures. The groupings became weaker, however, with increasing pig decomposition age (Figure 4a).

The number of insects collected on the first two days of sampling pigs (24–48 hours after placement, decomposition ages 1 and 2) was lower than on the subsequent two decomposition ages ( $F_{3, 38} = 7.27$ ,  $p = 0.0006$ ) (Figure 4b). Insect diversity was also low with five taxa trapped on these pigs, but only three representing the vast majority of newly arriving insects (*Lucilia coeruleiviridis*, *Lucilia illustris*, and *Phormia regina*). Samples from decomposition ages 3 and 4 were much more diverse, but were again dominated by the same

three species, with the addition of a late dominant species, *Cochliomyia macellaria*, on day 3 pigs. Beetles, while low in abundance, were collected only on day 2–4 pigs.

The relative abundance of taxa changed over the four day experiment. The relative abundances of *L. coeruleiviridis* and *L. illustris* were high on day 1 and declined over the next 2 days, whereas the relative abundances of *P. regina* and *C. macellaria* increased concomitantly (Figure 4c). Surprisingly, this pattern reversed on the fourth day of decomposition, on which the relative representation of these three taxa was similar to day 1.

Because the pigs were sampled daily for four days in the decomposition field before they were relocated to the transfer field, we also conducted linear discriminant analysis of insect community structure on these pigs only on the day of transfer to minimize the effects of climate variation and to enable a direct comparison to the transfer field on the same day. Results showed significantly different community structures, indicated by the lack of overlap of their 95% confidence ellipses, for the four decomposition ages sampled simultaneously on the same day (Figure 5a). Although differences between the two trials (September and October) contributed to variation, this pattern held for both trials, as pigs in the two trials grouped with the respective pigs of the same decomposition age.

Like the patterns observed across all four sampling days of the experiment, those from the transfer day alone showed dominance of *L. coeruleiviridis*, *L. illustris*, and *P. regina* on all pig decomposition ages, as well as dominance of *C. macellaria* on day 3 pigs. Again, day 1 pigs had lower insect diversity and significantly lower abundance than any other decomposition age, and Coleoptera was sampled from day 2–4 pigs. Mean abundance

of insects did not differ significantly in a comparison of all four sampling days and the transfer day only in the decomposition field ( $t_{42} = 1.2328$ ,  $p = 0.2245$ ). The relative abundances of the four major taxa (*L. coeruleiviridis*, *L. illustris*, *P. regina* and *C. macellaria*) were also similar on the day of transfer and the preceding 3 days. Therefore, the community composition on a single day (transfer day) in the decomposition field satisfactorily represented the asynchronous succession in the same field during the previous 3 days.

***Succession of the insect community in the transfer field*** After relocation of the decomposing pigs of different ages to a nearby field, the clear separation in linear discriminant analysis that we observed in the decomposition field became much weaker with broad overlap among different decomposition ages (Figure 6a). The greatest overlap was between day 1 and day 2 pigs, whereas day 3 pigs separated significantly from day 1 and 2 pigs. There was no significant difference in the number of insects sampled by pig decomposition age in field 2 ( $F_{3, 14} = 1.176$ ,  $p = 0.3540$ ).

***Insect arrival in the decomposition field vs. transfer field*** More insects were trapped and hand-collected in the decomposition field ( $93.7 \pm 17.3$  insects) than in the transfer field ( $51.3 \pm 11.7$  insects) ( $t_{30} = 2.0289$ ,  $p = 0.0514$ ) across pigs of all decomposition ages on the transfer days. A higher diversity of insects was also sampled in field 1, with several rare species (*Calliphora vicina*, *Chrysomya rufifacies*) only sampled on pigs prior to relocation. The numbers of coleopterans sampled were low (<10 total) in both fields across pigs of all decomposition ages on the transfer days. The relative abundance of taxa over decomposition

was different in field 2. The pattern of change of the three major taxa was less consistent than in field 1, but the increase in *C. macellaria* mirrored the increase in field 1.

Insect diversity sampled from day 1 pigs after relocation was higher than earlier in field 1. *Lucilia sericata*, *Cochliomyia macellaria*, and Sarcophagidae were sampled on pigs after relocation in field 2, although overall numbers and relative percentages of these taxa on day 1 pigs were low. Greater insect diversity after transfer occurred only with day 1 pigs. The relative abundance of the three major taxa also varied between the decomposition and transfer fields. *L. illustris* was equally represented in the decomposition field throughout the experiment, including on the transfer day, representing ~22% of the three major taxa (Figures 4c, 5c). After translocation however, *L. illustris* represented slightly more than 10% of the insect diversity, as the relative abundance of *P. regina* was higher in field 2.

Unlike with the day 1 pigs, a lower diversity of insects was sampled from day 2 pigs after relocation. Three taxa, *Lucilia cuprina*, *C. rufifacies*, and several coleopterans, were sampled in the decomposition field but were not sampled on the same pigs after they were transferred to field 2. The relative percentage of *L. illustris* sampled from pigs after relocation was also lower than in field 1 (Figure 6c).

Day 3 pigs had very similar community structures across the two fields. Only one taxon, *C. rufifacies*, was sampled in field 1 but not in field 2. Relative percentages of taxa on pigs were also very similar across field for day 3 pigs, with *P. regina* having the highest representation, and other taxa like *L. sericata* and *L. cuprina* in low relative abundance.

Day 4 pigs attracted similar taxa across fields in terms of the relative representation of the most abundant taxa, but they differed slightly in terms of diversity of rare taxa. One species, *C. vicina*, was sampled on day 4 pigs in field 1 but was not sampled after their relocation to field 2. As in field 1, the pattern of insects on day 4 pigs in field 2 was inconsistent with the pattern of changes in relative abundance seen in days 1–3.

## **Discussion**

Complex biological processes that are climate-dependent and site-specific, like decomposition and ecological succession, are subject to inherent variability [43]. To minimize variation in our study, we ensured that pigs were of the same size (~1.5 kg) and temperature ( $\leq 0^{\circ}\text{C}$ ) at their time of placement in the field, as the rate of decomposition is highly influenced by mass and temperature [44]. We also decreased potential sampler bias by incorporating a passive sampling method, the vented-chamber, into our sampling protocol.

While we sought to minimize environmental influences, several factors contributed substantially to variation in our study, including (1) differences between the two fields, (2) daily variation related to the number of days that each pig spent in the decomposition field, and (3) seasonal variation. In selecting two similar fields for this study, we considered proximity, vegetative characteristics, and level of sun exposure to be the most important factors to keep constant across fields. Insect succession differs between shaded and full sun exposure, as some necrophilous insects like *L. illustris* are more commonly sampled from sun-exposed carrion [5, 40], and silphid and staphylinid beetles are more likely to be sampled

from shaded carcasses [45]. We ensured that sun exposure was consistent across fields in the locations where pigs were placed. We also aimed to keep vegetation in the two fields similar, as populations vary based on habitat type, perhaps even on a microgeographic scale [28, 29]. Proximity of the two fields ensured minimal differences in their microgeographic characteristics.

The length of time each pig spent within the decomposition field was another source of variation. Because pig placement was staggered over four days, more successive observations were made on day 1 pigs than on day 4 pigs (Figure 4a). When data points from all five days of the experiment were used in LDA, there was not clear separation among pig decomposition ages. Samples from day 1 pigs were represented over 4 days of the experiment, contributing to substantial variation in the trapping results. This variation was largely eliminated when succession was assessed only on the last day (transfer day) across pigs of different decomposition ages (Figure 5). Ambient temperature increased  $>5^{\circ}\text{C}$  during the four days in the decomposition field in September and varied  $>5^{\circ}\text{C}$  in October, further contributing to variation in trapped insects (Figure 3).

Seasonal differences (September versus October) probably contributed most to variation. The rate of decomposition is greatly influenced by ambient and soil temperature [21]. Three replicates were conducted in late September, when mean ambient temperature was  $\sim 22^{\circ}\text{C}$ , and two replicates were in mid-October under ambient temperatures of  $\sim 10^{\circ}\text{C}$ . Thus, decomposition and succession proceeded much faster in September. Combining these replicates resulted in greater variance, particularly in late stages of decomposition – day 4 in

September represented a substantially more advanced stage of decomposition than day 4 in October. This was evident in the apparent “reversal” of the successional pattern on day 4 (Figures 4c, 5c) where declining representations of *L. coeruleiviridis* and *L. illustris*, and increasing representation of *P. regina* and *C. macellaria* were reversed on day 4. Closer examination of the data revealed that while the September replicates trapped more insects than the October replicates (September: 1,695 insects on 3 pigs, 565/pig; October: 921 on 2 pigs, 460/pig), the October replicates caught substantially more insects on day 4 than the September replicates in both fields. In both fields ~88-fold more insects were trapped in October than in September (decomposition field: 353 vs. 3, transfer field: 87 vs. 1). Since succession in October was substantially slower, day 4 in October likely corresponded to an earlier day in September as evidenced by trap catches and community structure. In addition, some cool weather species like *Calliphora vicina* were found only in the October replicates.

To minimize these sources of variation, we focused our analyses on the day of transfer only. It is important to note however, that while daily temperature variation was minimized and all pig decomposition ages received the same number of observations, the seasonal effect remained a major source of variation in this analysis as well. When the transfer day alone was analyzed in LDA, there was clear separation among pig decomposition ages in the decomposition field (Figure 5a). Taxa representations were strikingly similar between the four days of trapping in the decomposition field (Figures 4b,c) and the transfer day only (Figures 5b,c), justifying a comparison of the decomposition and transfer fields on a single day.

While the community structure differed by pig decomposition age on the day of transfer in the decomposition field, the distinct separation among pig decomposition ages was not as well defined after the pigs were moved to field 2 (Figure 6a). The community structures on day 1 and day 2 pigs were not significantly different. Surprisingly, neither was the difference in structure of day 1 and day 4 pigs, despite their decomposition age differences and significant separation in field 1. This “reversal” on day 4 to a pattern reminiscent of earlier decomposition stages again highlighted the dominance of the October replicates, which represented a much slower decomposition rate. LDA became less effective for assessing community structure because our sample size declined while seasonal variation increased.

Considering only the transfer day, generally more insects were trapped per pig in the decomposition field than in the transfer field. However, the relative abundance of taxa varied more substantially between the two fields. In the decomposition field, we observed the expected pattern of declining relative abundances of *L. coeruleiviridis* and *L. illustris*, and increasing representation of *P. regina* and *C. macellaria* between days 1 and 3 (Figure 5c). In the transfer field, on the other hand, the only clear pattern was an increase in *C. macellaria* (Figure 6c).

As indicated earlier, decomposition progressed more rapidly in September. Thus, total trap catch per pig in the decomposition field varied from 21.3 on decomposition age 1 pigs to 131.0, 172.3 and 3.0 on days 2, 3, and day 4, respectively. In October, the corresponding numbers were 27.5, 38.0, 112.5 and 176.5. Clearly, pigs in their fourth day of

decomposition in October were still in a rather early decomposition state as indicated by the large numbers of insects trapped and the early successional community structure. Because LDA was conducted on the absolute trap catch, the day 4 October catches dominated the dataset.

*Calliphora vicina* and *Chrysomya rufifacies* were trapped only in field 1, and in low numbers (<10). This is the first record of *C. rufifacies*, the hairy maggot blow fly, in central NC. This invasive fly species poses significant challenges to forensic entomologists because its predatory larvae can consume other first responders and thus create a false picture of succession. This can lead to potentially incorrect postmortem interval estimations. The unique feeding behavior of *C. rufifacies* may also affect the arrival pattern of native insects, further complicating PMI estimations [35].

From our analyses, we are able to draw several conclusions. First, the inherent variability in decomposition and ecological succession creates a challenge as climate changes over the course of the experiment. Although we attempted to minimize several sources of variation in our experimental design, seasonal differences dominated the variation. This effect can be mitigated with larger sample sizes in a single replicated experiment conducted during a minimum number of days. Second, it was clear that different insects were differentially attracted to pigs of different ages of decomposition. Differential attraction was also observed in the transfer field after relocation but was less pronounced. Third, the general community structures of trapped insects on decomposing pigs were similar before and after relocation, with the exception of several rare taxa, indicating that the predicted insect

succession continues in locations that are geographically and ecologically similar. All major and some minor (i.e., rare) taxa were sampled from pigs in both fields, although a few rare taxa were only found prior to relocation. Fourth, necrophilous insects locate a newly relocated host within minutes, guided by olfactory cues. Insects were sampled from pigs of all four decomposition ages in field 2 within 15 minutes after placement. Although other studies have documented necrophilous insect host location within minutes [10, 46], our design excluded all sensory modalities but olfaction. While other cues, such as on-carcass interactions or even visual cues may be important for insect oviposition, we focused solely on olfactory attraction with the use of the vented-chamber, as it sampled insects before they were able to interact with the carcass. Finally, olfactory cues alone can shape the community structure of necrophilous insects. These differences may be related to differing VOC profiles from the carrion itself or from other insects on the decomposing body [47, 48].

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Table 1. Placement and sampling schedule for all pigs. Each pig was assigned a unique number. The decomposition age of each set of pigs (in days) is indicated in parentheses.

Experiment day	Pigs placed in decomposition field	Pigs sampled in decomposition field (age)	Pigs sampled in transfer field (age)
September 2015			
1	1, 2, 3	none	none
2	4, 5, 6	1, 2, 3 (day 1)	none
3	7, 8, 9	1, 2, 3 (day 2) 4, 5, 6 (day 1)	none
4	10, 11, 12	1, 2, 3 (day 3) 4, 5, 6 (day 2) 7, 8, 9 (day 1)	None
5 (transfer)	none	1, 2, 3 (day 4) 4, 5, 6 (day 3) 7, 8, 9 (day 2) 10, 11, 12 (day 1)	1, 2, 3 (day 4) 4, 5, 6 (day 3) 7, 8, 9 (day 2) 10, 11, 12 (day 1)
October 2015			
1	13, 14	none	None
2	15, 16	13, 14 (day 1)	None
3	17, 18	13, 14 (day 2) 15, 16 (day 1)	None
4	19, 20	13, 14 (day 3) 15, 16 (day 2) 17, 18 (day 1)	None
5 (transfer)	none	13, 14 (day 4) 15, 16 (day 3) 17, 18 (day 2) 19, 20 (day 1)	13, 14 (day 4) 15, 16 (day 3) 17, 18 (day 2) 19, 20 (day 1)

Table 2. Forensically significant insects sampled from all pigs and both experimental fields. If no species listed, taxonomic identifications ended at the family or genus level. Taxa sampled in the decomposition field are denoted with a \*, and taxa sampled in the transfer field are denoted with a †.

Order	Family	Genus and species
Diptera	Calliphoridae	<i>Lucilia illustris</i> (Meigen)* †
		<i>Lucilia coeruleiviridis</i> (Macquart)* †
		<i>Lucilia sericata</i> (Meigen)* †
		<i>Lucilia cuprina</i> (Wiedemann)* †
		<i>Phormia regina</i> (Meigen)* †
		<i>Cochliomyia macellaria</i> (Fabricius)* †
		<i>Calliphora vicina</i> (Robineau-Desvoidy)*
Coleoptera	Sarcophagidae	<i>Chrysomya rufifacies</i> (Macquart)*
	Sarcophagidae	Sarcophagidae spp. *†
	Muscidae	<i>Musca domestica</i> (Linnaeus)* †
	Silphidae	<i>Necrophila americana</i> (Linnaeus)*
	Histeridae	Histeridae spp.*
	Staphylinidae	Staphylinidae spp. *†
	Dermestidae	<i>Dermestes</i> spp. *

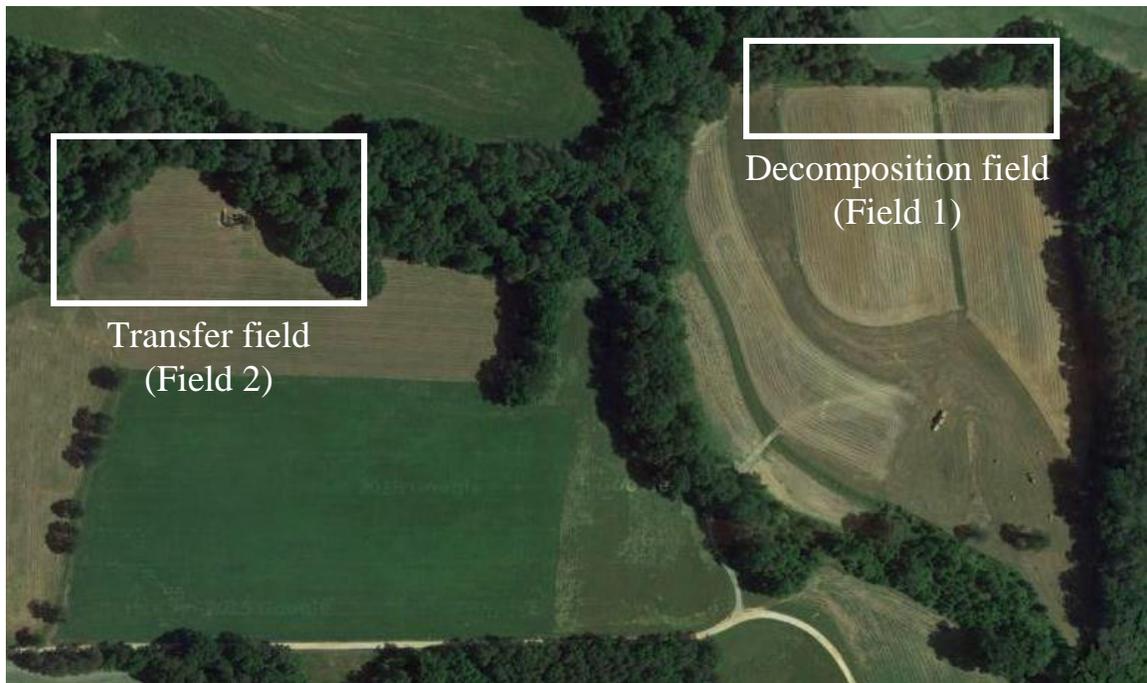


Figure 1. Experimental fields at the Lake Wheeler Road Field Lab in Raleigh, NC. Pigs were added daily to the decomposition field (field 1). Pigs were sampled in this field daily, beginning 24 hours after their initial placement in the field. On the fifth day of the experiment, all pigs were relocated to the transfer field (field 2) and sampled.

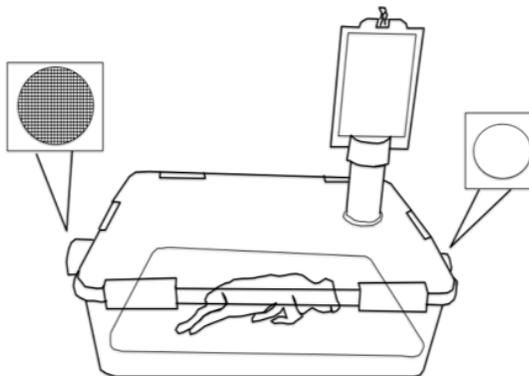


Figure 2. The “vented-chamber” method, as described in Chapter 3. The collection unit consists of a 39 liters airtight chamber with PVC ports. Ports on the left and top of the box, with the orientation as above, were kept open with mesh window screening. Thus, air could flow freely through the chamber, but insects could not enter it. The right port was capped. The top port, or chimney, opened to back-to-back unscented glue traps. Pig orientation in reference to the ports was always as shown above.

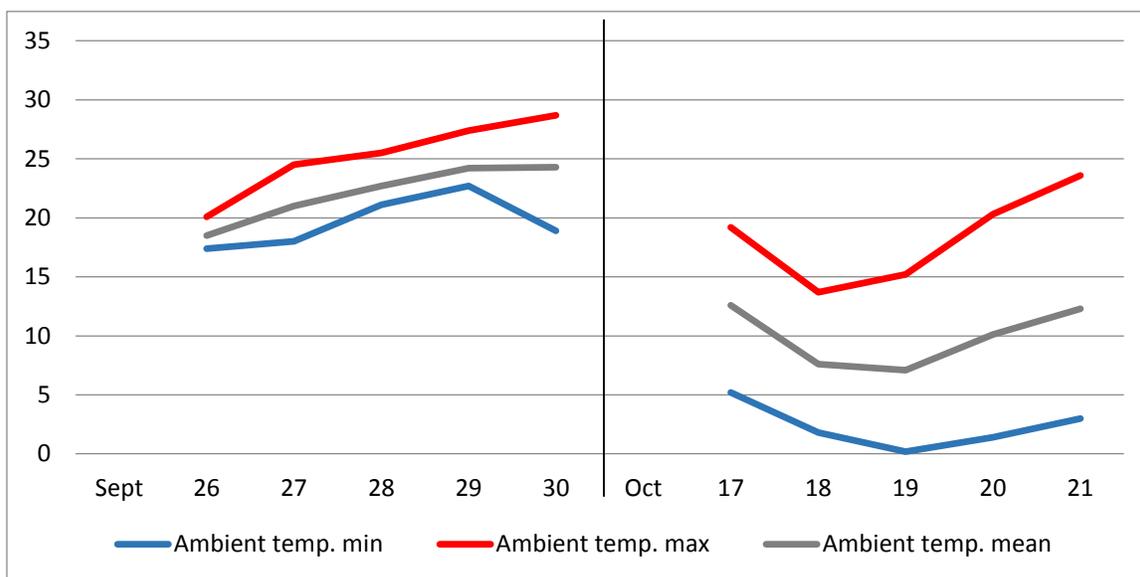


Figure 3. Daily minimum, maximum, and average ambient temperatures ( $^{\circ}\text{C}$ ) near the field research site during the experiment. Temperature data were acquired from the State Climate Office of North Carolina's Lake Wheeler Road Field Lab weather station. The average temperature during the September experiment was  $22.1 \pm 1.1^{\circ}\text{C}$  (SEM). Temperatures during the October experiment were much cooler, averaging only  $9.9 \pm 1.1^{\circ}\text{C}$ .

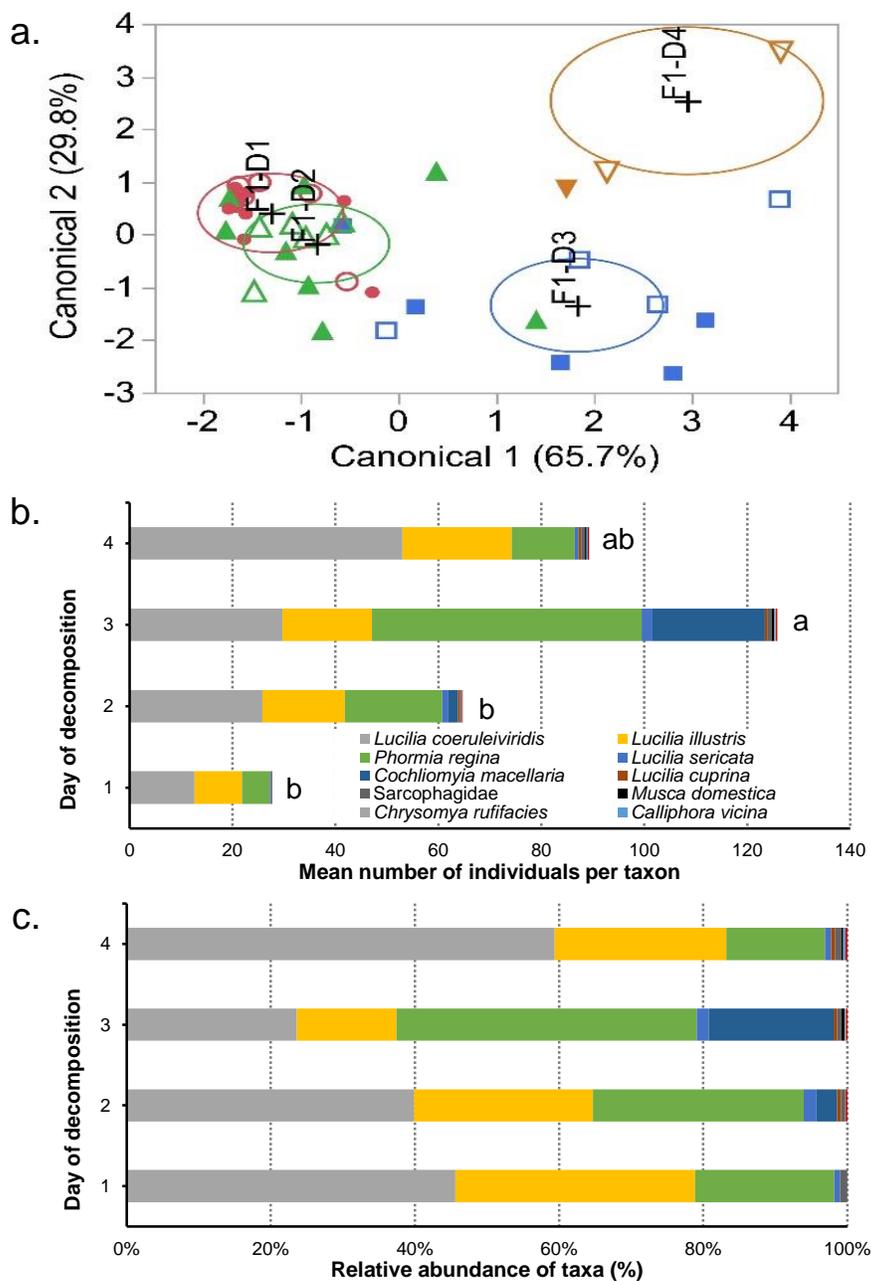


Figure 4. Insect activity in the decomposition field (field 1) across all days of the experiment.

(a) Linear discriminant analysis of community structure by day of decomposition (i.e., pig age) and season (September = solid symbols, October = open symbols). (b) Mean number

and (c) relative abundance of taxa trapped or hand-collected daily from pigs during the 4-day decomposition process. N = 20 pigs. In a, the percentage adjacent to each canonical variable represents the proportion of the sum of the eigenvalues. In b, unique letters indicate significantly different total mean insects by day using one-way ANOVA ( $F_{3, 38} = 7.143$ ,  $p = 0.0006$ ) and Tukey's HSD.

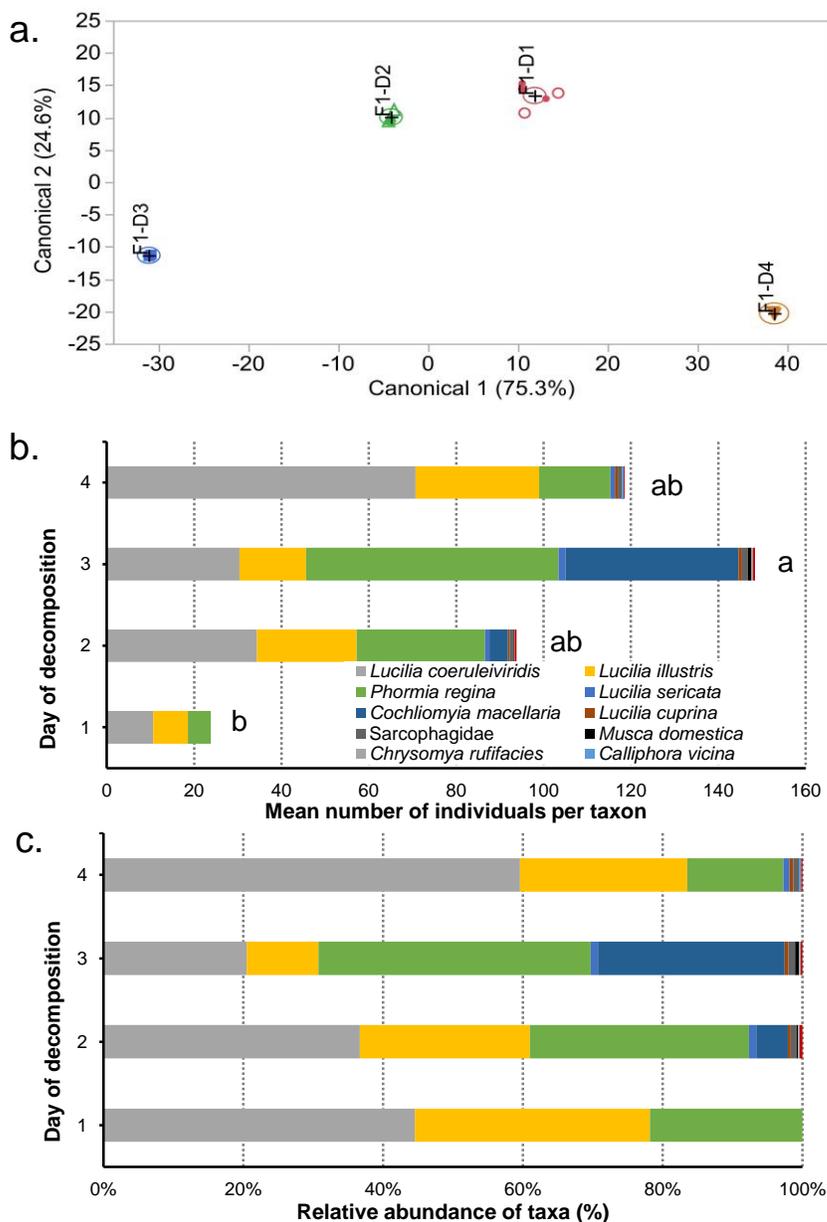


Figure 5. Insect activity in the decomposition field (field 1) on the day of transfer, just prior to pig relocation. (a) Linear discriminant analysis of community structure by day of decomposition (i.e., pig age) and season (September = solid symbols, October = open

symbols). (b) Mean number and (c) relative abundance of taxa trapped or hand-collected from pigs in field 1 on the day of transfer. N = 19 pigs. In a, the percentage adjacent to each canonical variable represents the proportion of the sum of the eigenvalues. In b, unique letters indicate significantly different total mean insects by day using one-way ANOVA ( $F_{3,14} = 3.8042$ ,  $p = 0.0348$ ) and Tukey's HSD.

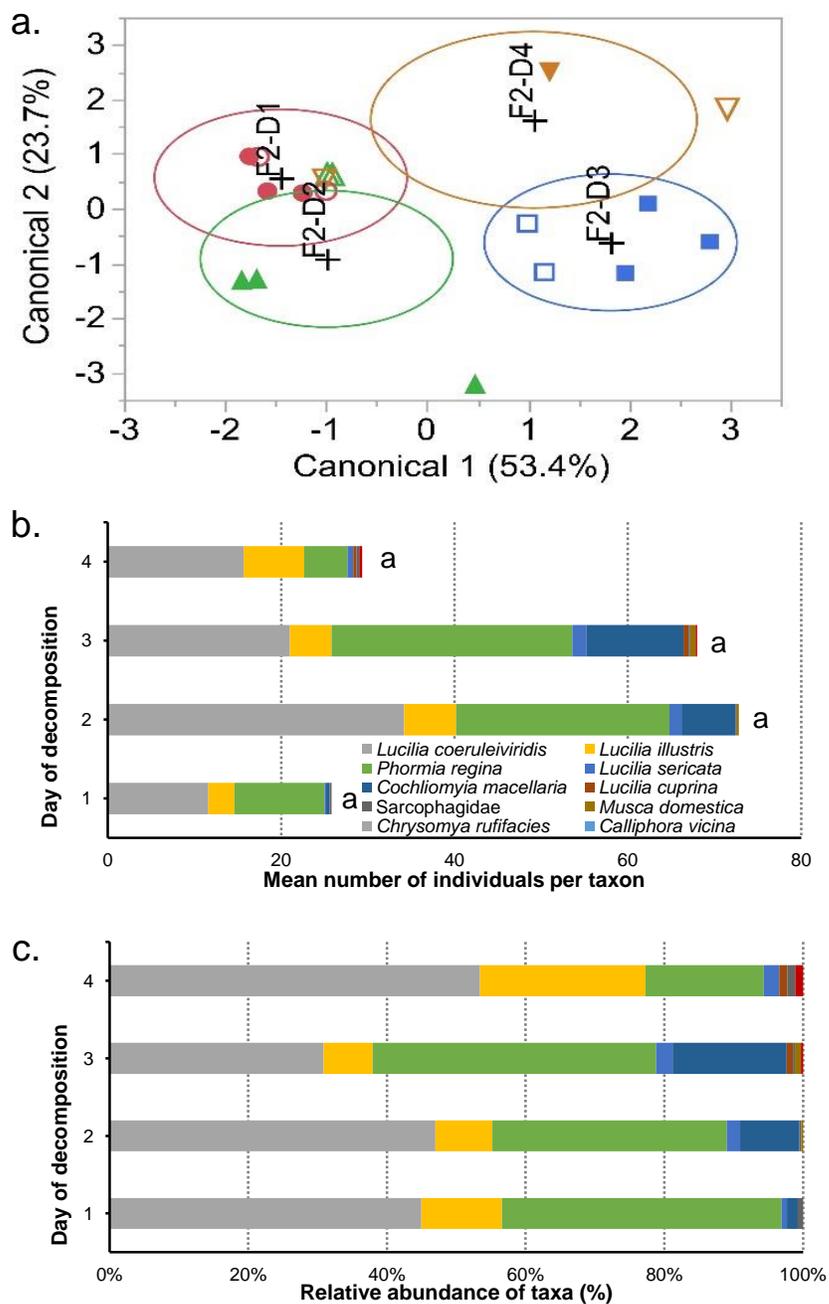


Figure 6. Insect activity in the transfer field (field 2) on the day of transfer, just after pig relocation. (a) Linear discriminant analysis of community structure by day of decomposition (i.e., pig age) and season (September = solid symbols, October = open symbols). (b) Mean

number and (c) relative abundance of taxa trapped or hand-collected from pigs in field 2 on the day of transfer. N = 19 pigs. In a, the percentage adjacent to each canonical variable represents the proportion of the sum of the eigenvalues. In b, the same letter indicates no significant differences in total mean insects by day using one-way ANOVA ( $F_{3, 14} = 1.176$ ,  $p = 0.3540$ ).

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