FINAL TECHNICAL REPORT

Report Title: Final Report: Development and Validation of Standard Operating Procedures for Measuring Microbial Populations for Estimating a Postmortem Interval

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Abstract

Predicting the postmortem interval of a decedent is a major task of law enforcement. Most methods implemented by death investigators rely on qualitative information (i.e., rigor mortis, livor mortis). Microbes represent 99% of somatic cells in and on a human body. No data are available on the use of these organisms to predict the time since death of a decedent, though it is known that certain chemicals, many of which are likely a result of microbial communities, are released by decomposing remains in a reliable pattern. Moreover, the effects of microbes on insect colonization of remains, sometimes the best predictor of a postmortem interval, are not understood. Because of a lack of understanding of microbial succession on decomposing human remains, no standard operating procedures (SOP) for sampling and using this information have been developed and validated. We developed a SOP for sampling and pyrosequencing bacteria communities on human remains to predict the actual time since death. We conducted a series of laboratory and field studies to achieve this end. The field studies will implement the SOP for sampling bacteria on decomposing human remains at the Forensic Anthropology Research Facility at Texas State University.

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EXECUTIVE SUMMARY

People that die represent nutrient rich resources for consumers, such as microbes and flies. Understanding the interactions between these groups could lead to a better understanding of the trophic level succession that occurs on remains, and potentially be used to develop and implement novel techniques (i.e., Biolog EcoPlates™ and pyrosequencing) for estimating the minimum postmortem interval (min-PMI) of a corpse.

The purpose of the proposed research was to develop Biolog EcoPlates™ and pyrosequencing (i.e., metagenomic sequencing technology) as methods used for determining the min-PMI of a corpse. Our research will elucidate multi-trophic interactions between carcasses, microbes, and blow flies (Insecta: Calliphoridae). We hoped to demonstrate that microbial communities undergo succession patterns and this process can be modeled and used to predict the min-PMI of a corpse with a known error rate and understanding of the temporal variation associated with this estimate. We attempted to conduct this research in a manner that would result in a standard operating procedure that can be used to make such estimations, while meeting the Daubert Standard.

RESEARCH DESIGN AND METHODS FOR DATA ANALYSIS

Objective 1. Microbial-arthropod successional interactions for estimating the PMI of a corpse. The structural and functional changes in microbial communities on decaying human remains and decaying human tissue in laboratory conditions will be described. We will identify specific microbial communities and their associated metabolic processes that influence the initial attraction, arrival sequence and interactions of blow flies to a decomposing resource.

Experimental Design. Three sets of human remains provided by the Forensic Anthropology Center at Texas State (FARF) at Texas State University in San Marcos, TX were placed minimally 15 m apart under naturally changing environmental conditions (ACC). An additional three sets of human remains were placed in blow fly exclusion cages (EXC). All sets were placed in a randomized block design. Studies were carried out during the fall of 2011, spring of 2012 and fall of 2012 due to the availability of procuring paired sets of remains.

Sampling Regime: Triplicate samples of the microbes were collected every 8 h for 5 d, which coincides with reduction in attraction of adult blow flies[1], from each of 5 areas (head region, anal/genital region, skin of the abdominal/thoracic region, soil samples directly underneath the remains, and soil samples 1 m away) (Figure 1). Sterile cotton swabs were used to collect microbial community samples from the remains, while a standardized sterile soil corer (~ 2.5 cm diameter) was used for collection of the soil samples. Samples from each of these sampling areas were divided for identifying the bacterial ecological, structural, and functional changes, thus, describing the bacterial succession occurring during carcass decomposition. Swab and soil core samples were collected using a sterile technique and stored at -80°C for first two cadavers, and at -20°C for rest four cadavers until processing for DNA extraction was completed (see below for DNA extraction). Each sample was uniquely identified and tracked throughout analysis, associating the location from which the sample was obtained and the time of the sample.

To understand abiotic factors hypothesized to influence microbial community succession and subsequent blow fly colonization, ambient weather conditions of temperature, humidity, rainfall, and sunlight were also monitored at hourly intervals using a standard weather station within 10 m of all carcasses. Temperature was monitored at proximate locations to each carcass: Micro-T temperature loggers made 0.25 h hourly 1m adjacent to the carcass. Climate conditions were statistically analyzed with microbial succession, timing of first oviposition (laying eggs), initial presence of dominant blow fly species, and subsequent species colonization and persistence throughout decomposition.

Objective 1 (a) Community Function (Physiological Profiles): The diversity of bacterial carbon resource utilization over time provide a physiological profile that can be used as a presumed signature and surrogate for the diversity of volatile odor production during early stages of decomposition[2]. Functional diversity changes of heterotrophic microbial communities on and within decaying carcasses were described by physiological profiles of each carcass microbial community through the decomposition process using readily available and established culture-based Biolog EcoPlate techniques[3-6].

Biolog EcoPlates™ were originally used in the medical field, but have found recent successful use in ecological studies[4,7,8], including recent investigations in both terrestrial[5,6] and aquatic habitats[7,9,10]. EcoPlates™ have 31 different carbon sources represented in triplicate, giving internal replication on a plate and were designed for understanding an entire microbial community.

These Biolog EcoPlates™ were used to trace the bacterial carbon source utilization profile over the course of decomposition. Biolog EcoPlates™ are colorimetric 96 well microtiter plate assays that contain a diverse array of carbon sources plus tetrazolium violet dye among the microtiter wells. When the carbon source of any well is utilized the tetrazolium dye is reduced resulting in color development in the well[4,11]. The diversity of color development associated with each plate is quantified using a plate reader (e.g., Tecan Sunrise™). The diversity of carbon source utilization for each plate provides a microbial metabolic community profiles (MMCPs) for that sample (*sensu*[3]). When samples from the same substrate are taken over time, this technique describes the MMCP succession of the microbial communities of that substrate and of the microbial community structure[6]. This was done according to recommendations and methods outlined by Stefanowicz[4] and Weber and Legge[12].

Objective 1 (b) Bacterial Community Structure: Each set of human remains was sampled every 8 h for 5 d after death (Figure 2 is an illustration of what was initially proposed for Biolog & Pyrosequencing Samples) to coincide with the natural decline of blow fly attraction human remains, during the summer in Texas[1]. Microbial community structure was evaluated using pyrosequencing techniques in order to identify microbial community members (specifically bacteria), community richness and diversity. Samples were collected as described by Costa-Martínez et al.[13].

DNA extraction: For pyrosequencing preparation, samples (listed under Sampling Regime) were manipulated using aseptic technique in a biological safety cabinet. DNA was extracted from each

sample and from 0.25 g soil samples using organic extraction method. Briefly, swabs or 0.25 gm of soil were placed in 2 ml tubes with zirconium bead (100 uM) (OPS Diagnostics, LLC, Lebanon, NJ, USA), 540 µl Tris-EDTA (pH=8) (Amresco LLC, Solon, Ohio, USA), 50 µl 10% SDS (Amresco LLC, Solon, Ohio), 10 µl proteinase K (20 mg/ml) (Amresco LLC, Solon, OH, USA), 4 µl of RNaseA (100 mg/ml) (Amresco LLC, Solon, OH, USA), 100 µl NaCl (5M), and 80 µl CTAB extraction solution (TEKNOVA, Hollister, CA, USA), and then samples were thoroughly homogenized using FastPrep®-24 homogenizer (MP Biomedicals, USA) and incubated overnight at 65°C. Sequential extraction in a 1X volume was performed using phenol (pH=8.0), phenol/chloroform/isoamyl alcohol (25:24:1), and chloroform/isoamyl alcohol (24:1) by centrifugation at 6000 x g for 6 min. DNA was precipitated in 0.7 volume of isopropanol, washed in 70% ethanol, dissolved in nuclease free water, and quantified by NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). DNA products from each sample were aliquotted and sent to the Research and Testing Laboratory (http://www.researchandtesting.com/) for 16S rDNA 454-pyrosequecning using universal bacterial primer pair 27F (5'- GAGTTTGATCNTGGCTCAG) and 519R (5'- GTNTTACNGCGGCKGCTG) by bacterial tag-encoded FLX-Titanium pyrosequencing (bTEFAP) method [14] in Genome Sequencer FLX System (Roche, Nutley, NJ, USA). All FLX related procedures were performed following Genome Sequencer FLX System manufacturers instructions (Roche, Nutley, NJ, uSA).

Post-sequencing data processing: Sequencing error was minimized using PyroNoise[15] as implemented in Mothur v. 1.29[16]. Low quality regions of the sequences were trimmed using sliding window (50 bp; Q35) option in Mothur v 1.29[16]. All sequences were checked for chimera formation using Uchime[17] as implemented in Mothur v. 1.29[16], and using most abundant sequence as reference data. Suspected chimeras were deleted and rest sequences were utilized for further analyses. Hierarchical classification of 16S rDNA bacterial sequences were done using Naïve Bayesian rRNA classifier version 2.2[18] as implemented in Mothur v. 1.29[16]. Only sequences having ≥ 80% bootstrap support were considered classified at a particular hierarchical level. OTU based Simpson and UniFrac diversity indices were calculated in Mothur v. 1.29[16]. To avoid spurious OTU count because of different number of sequence reads in different samples, sequences were normalized by picking same number of sequence reads from each sample, before α- and β-diversity and richness estimations. Samples were clustered using OTU based distance matrix by non-metric multidimensional scaling (NMDS) analyses in Mothur v. 1.29[16]. NMDS data from first three axes for all treatments were plotted using rgl package in R version 2.15.1[19]. Analysis of molecular variance (AMOVA) was used to test whether spatial separation between different clusters in multivariate analyses is statistically significant or not. OTUs responsible for significantly temporal shift of microbes with time after placement of cadavers were determined by measuring indicator species analyses using Mothur v. 1.29[16]. Although, microbes can survive to wide range of temperatures, even below freezing[20], a minimum base temperature of 0°C was used for calculation of accumulated degree hours (ADH). This assumption was based on our knowledge of psychrotrophic and mesophilic bacteria, which were common in our previous research[21], and are not expected to grow in freezing conditions.

Objective 1(c) Develop a standard operating procedure for analyzing and interpreting the microbial community samples taken from the human corpse and estimating the PMI of the

remains. Methods developed from the previous sub-objectives will be compiled and made available to the forensic community through publications in refereed journals.

Objective 2. Conduct lab validation studies to quantify variation associated with microbial succession on human tissue. We proposed to conduct the following research; however, we had difficulty receiving the human remains needed for the first objective. In fact, we did not conduct our final trial until fall 2012. And we are still analyzing data associated with this trial. Consequently, we were unable to identify the key bacteria consistently present across humans in each trial which would allow us to conduct this objective (though we currently have all the necessary data to begin doing so now). However, we were able to address aspects of this issue by altering our research path slightly to allow us to answer the same questions but with different methods. Please see the results section for a description of the data gathered in support of this objective.

Here are the original methods that were proposed in our grant. Please proceed to page 13 for updated methods.

2 (a) Abiotic factors affecting select microbes: It was our goal to conduct controlled observations of bacterial communities associated with decomposing human remains in order to clarify results obtained from field observations. We intended to use a select set of bacteria species identified in 1 (b) and use them in a series of experiments conducted to determine the effects of humidity (25% and 75% RH) and temperature (20°C and 34°C) on their proliferation on human tissue. As an example, we find *Proteus* species commonly associated with *L. sericata*[22], which are similar to strains implicated in the elimination of other microbes on a wound colonized by flies[23]. The growth of four key strains was to be evaluated on human tissue over time in three replicates of each of the four possible combinations of humidity and temperature to determine abiotic influences on key players in the microbial communities observed in the first objective. Bacterial strains were to be chosen based on their potential to be a temporal marker of human decomposition, high abundance, and prevalence across all cadavers studied in Objective 1. Human tissue will be seeded with a standardized number of bacterial cells, which was to be determined experimentally for each species to ensure that the microbes do not overpopulate the resource during the duration of the experiments, in each of the environmental combinations. The standardized number of cells would represent an ecologically relevant density. Then five samples were to be taken from five sites on the tissue and pooled over the course of 72 h. Three tissue samples were to be used as replicates. Quantitative PCR was to be conducted on each sample to determine the concentration of bacteria in a standard volume removed from the tissue. CFUs would have been determined and the $\log_{10}$ change after exposure calculated. In addition, samples collected for quantitative PCR were to be applied to Biolog plates (as described above) to observe the effects of these key microbes on community function and how the abiotic factors studied will affect those functions.

Microbe communities that the study bacteria will be cultured with were to be evaluated to determine effects of variation in microbe community on their growth. Samples were to be taken from the tissue at the beginning and end of each experiment to determine the effects of 1) the initial community structure on growth of the four target species and 2) the effect of the study

species on ultimate community structure. Pyrosequencing methods would have been done as noted above.

2 (b) Biotic factors affecting select microbes: The effects of *C. rufifacies* and *L. sericata* (eggs, larvae, adults) on bacteria communities associated with decomposing human tissue was to be quantified. Methods for raising sterile larvae were to be adapted from Sherman and Tran[24]. The Tarone and Tomberlin laboratories are currently capable of rearing sterile flies, which do not demonstrate microbial growth after 72 h of incubation of larval tissue in sterile medium. A single clutch of eggs was to be agitated in 1% sodium sulphite for 10 min and surface sterilized per Ahmad et al.[25]. Eggs would then be rinsed with saline, placed in sterile containers, and fed previously prepared sterile liver media. Pupae would then be removed, sterilized using methods previously described, placed in sterile petri dishes in a growth chamber. Emergent flies would then be placed in sterile enclosures, provided sterile food *ad libitum*. Eggs were to be collected using the sterile liver:agar mixture. Resulting eggs were to be used in the following experiments after surface sterilization.

Fly Colonies. Approximately 1,000 adult *L. sericata* and *C. rufifacies* respectively were to be sampled from decomposing animal remains located in College Station, TX to get a sufficient genetic sample. Cages were to be maintained in the laboratory at 27°C and 60% RH. Water with 50% sugar concentration was to be provided. Cages, sugar water, and consumables would be autoclaved prior to use.

Experiment 1. Parallel comparison of bacterial communities growing on human tissue when exposed to L. sericata *and* C. rufifacies*.* The four aerobic bacteria identified above were to be examined in this study. All human tissue used in the experiments described in Objective 2a would have been split between 2a and 2b to allow direct comparisons between experiments. For each bacterium/blow fly combination, the bacterial species was to be spiked onto the tissue (Table 1) and raised in the temperature and humidity condition from Objective 2a that is most relevant to typical environmental conditions near College Station, TX (i.e., the high RH, high temperature condition described in Objective 2a). All analyses done in Objective 2a was also to be conducted in this experiment, to enable a comparison of microbe communities in the absence of blow flies to those in their presence.

Experiment 2: Significance of bacteria-larval interactions on larval development. Entomological evidence is a potential data source informative of a min-PMI. It is entirely possible that different bacteria associated with human remains may alter the development rates of blow fly species. Accordingly, knowledge of microbial influences on blow fly development will be useful in identifying factors that affect error in entomologically based min-PMI estimates. Effects of direct contact of bacteria on fly development were to be assessed.

Growth curves were to be conducted on each bacterial species to determine the optimal starting concentration and sampling frequency, so as not to overgrow during the three day experiment. Individual bacteria (A&B represent two bacteria species from *L. sericata* and C&D represent bacteria species from *C. rufifacies*) were to be plated on appropriate agar at two different concentrations, appropriate to their growth rate (e.g. 10^2, 10^4 cfu/ml) and incubated at 34°C. Three replicates of each treatment previously listed were to be conducted for a total of 144 plates

per fly species. Sterility of eggs was to be confirmed prior to treatment with experimental bacteria. For each treatment, at least fifteen eggs from the identified blow fly species was to be obtained from colonies previously described, surfaced sterilized (see above) and placed on each replicate. Per Ahmad et al.[25], plates would be placed in a growth chamber set at 34°C and monitored daily for larval mortality and pupation. For the flies, immature survivorship, pupal weight, and development duration was to be recorded. Emergent adult weight would also be recorded. A sample was to be taken at each stage of development (eggs, larvae, pupae and adult flies) for determination of bacterial concentrations by serial dilution.

Data Analysis: Geometric mean, 95% confidence interval, of bacterial colony forming units would be determined. ANOVA was to be used to analyze CFU, larval survival and fly species interaction[26]. Least Significant Difference (LSD) test was to be used following a significant ($P < 0.05$) F test to separate means[26]. All percent data would have been arcsine transformed prior to analysis.

Implications for Criminal Justice Policy and Practice

Objective 1. Objective one was a survey of bacterial colonization associated with decomposing human remains. Pyrosequence analysis of bacterial DNA present at specific sites coupled with their physiological profiles on the remains will allow a comprehensive survey of the wealth of species colonizing these resources. These results will help us identify specific stages of bacterial community succession for more specifically targeted studies targeted at the bacteria most important to predicting a min-PMI and of microbial-insect interactions relevant to PMI estimates. The analysis will have a significant impact by identifying the diversity of bacterial species utilizing the remains, the stability of such microbial communities, by correlating the rate of decomposition with the insects and microbes present to the PMI of the remains, and by establishing if the porcine model (which we are currently analyzing) is appropriate for microbial studies of human decomposition.

Objective 2. Data produced could provide insight into biotic and abiotic factors affecting microbial communities and potential microbial markers of PMI. Factors affecting error rates of PMI predictions based on microbe and blow fly evidence will be identified.

MAIN BODY OF THE FINAL TECHNICAL REPORT

STATEMENT OF PROBLEM

Predicting the postmortem interval of a decedent is a major task of law enforcement. Most methods implemented by death investigators rely on qualitative information (i.e. rigor mortis, livor mortis). Microbes represent 99% of somatic cells in and on a human body. No data are available on the use of these organisms to predict the time since death of a decedent, though it is known that certain chemicals, many of which are likely a result of microbial communities, are released by decomposing remains in a reliable pattern. Moreover, the effects of microbes on insect colonization of remains, sometimes the best predictor of a postmortem interval, are not understood. Because of a lack of understanding of microbial succession on decomposing human remains, no standard operating procedures (SOP) for sampling and using this information has been developed and validated.

LITERATURE REVIEW

Determining the PMI (PMI) is not new to the forensic sciences[27] Knowing the time of death of a corpse is a primary objective during a criminal investigation as such information can eliminate or implicate individuals as suspects in cases of foul play. Most attempts to provide an estimate of PMI early in the decomposition process (i.e., 72 h) for corpses are based on evidence associated with the corpse itself. Methods hinge on a variety of factors. Some are highly suspect (i.e. eye witness accounts), while others tend to be remarkably accurate, but are time consuming or limited due to a lack of experts trained in the discipline required (i.e. entomology).

PMI can also be important in other instances outside of criminal investigations, such as settling estates and determining life insurance matters. However, as pointed out by Di Miao and Di Miao[27], many of the methods employed lack reliability and accuracy[28-30]. Adelson[31] emphasized the difficulty in estimating the PMI "beyond a reasonable doubt" necessary for litigation. Providing an estimate of the range with statistical relevance is also a challenge. If one examines the techniques employed, even those that are able to provide a range for the PMI, one will find that the variation increases as the interval from time of death to discovery increases[32,33]. Furthermore, many of the techniques currently employed lack validation or defined error rates. Consequently, these techniques, regardless of their admittance into a court of law, fail to meet the Daubert Standard[34] or the newly released parameters outlined by the National Research Council (NRC)[35].

Methods for estimating the PMI of a corpse typically rely on what is "in" or "on" the decedent at the time of death. These methods examine materials in the remains (i.e., physical, physiological, and biochemical) typically measuring rate and concurrency. Rate is a dynamic process measuring the change over time of a particular variable, dependent on biotic and abiotic factors[36]. Examples include, but are not limited to, phosphate levels in the decedent's blood serum[37] liver mortis, rigor mortis, algor mortis[38], and insect succession[33]. Other methods rely on information associated with the decedent and his/her activities leading up to disappearance, death and discovery. These data could include, but are not limited to, video surveillance, credit card records, or phone use.

Change in body temperature (i.e., algor mortis) is a common method employed to estimate a temperature-PMI (T-PMI) of a recently deceased individual[39]. While this technique has been in the literature since the 1800s, it was not until 1962 that this method was first modeled by Marshall and Hoare[40]. The initial model proposed focused on examining rectal temperature as it relates to time of death[40-42]. Since this initial work, data have been collected for estimating the PMI based on temperature changes in various organs including the skin, trachea, and brain[43-46]. Regardless of the site, these methods employ mathematical formulas (i.e., algorithms) to predict the rate of cooling of the remains; however, a number of confounding variables introduce variation and error in these estimates[47,48]. These variables include location of the remains (i.e., indoors vs. outdoors), exposure to external heating or cooling sources, physical condition of the individual[39] and inconsistencies inherent in the models themselves[40]. Furthermore, recording temperature runs the risk of damaging the corpse. Unfortunately, these estimates exhibit limited accuracy within approximately 6 to 50 h postmortem[49].

Changes in chemical composition of natural body fluids can be used to estimate the PMI during the early stages of decomposition[30]. Vitreous humor provides an accurate method for estimating

the PMI during the early stages of decomposition[50]. However, physiological differences between individuals posed a difficult hurdle in terms of recognition in variation exhibited by each person postmortem[49] with estimates not accounting for this variation resulting in over estimates of the PMI[51]. Consequently, the mathematical models employed have been revised to increase accuracy[52]. Modifications in standard operating procedures regarding the collection and analysis of these fluids has also increased the accuracy of current models for estimating the PMI[53].

Staining muscle (i.e. skeletal, smooth, and cardiac) tissue removed from the decedent has also been used to provide a PMI estimate[51]. Chandran[54] conducted research examining the use of staining smooth muscle from the iris of the decedent to determine its PMI. However, this area of research has produced mixed results and is still under debate[55]. Other areas involving rigor mortis and the rate at which muscle loses electrical reactivity. Rigor mortis, or stiffening of the muscle, is one of the most common methods used for estimating the early PMI. Lividity, or discoloration of the skin, is also a common method used to estimate the early PMI. However, this feature, much like rigor mortis, is a qualitative measure and the degree of error or variation is not known. These estimates are general and provide only some guidance for an investigation[31,56] during the initial 24 h after death[57]. Other methods involving blood chemistry [58]and motility of bone marrow cells[59] also are useful; however, these methods, again like many of the other methods discussed, are useful only during the initial few days after death.

Methods previously described only are suitable for remains within approximately 72 h of death. Once the body begins to decompose, other methods such as entomology, botany, and anthropology are used. Unfortunately, these methods rely on the assistance of experts with extensive training in the select field. And, in most cases, crime laboratories do not employ these experts full time and retaining the services of these individuals can be expensive and time consuming. Consequently, in many cases, the PMI estimate based on these other techniques is not incorporated into the investigation.

People that die represent nutrient rich resources for consumers, such as microbes and flies. Understanding the interactions between these groups could lead to a better understanding of the trophic level succession that occurs on the remains and potentially the development and implementation of novel techniques (i.e., Biolog plating and pyrosequencing) for estimating the PMI of a corpse. The purpose of the proposed research was to develop the use of Biolog plating and pyrosequencing (i.e. metagenomic sequencing technology) as methods for determining the PMI of a corpse.

Our research attempted to elucidate multi-trophic interactions between carcasses, microbes, and blow flies. We attempted to demonstrate that microbial communities do experience succession and that this process can be modeled and use to predict the PMI of a corpse. Our goal was to conduct this research in a manner that would result in a standard operating procedure that could be used to make such predictions while meeting the Daubert Standard.

Ecology of Decomposition. Animal carcasses, such as human remains, are nutrient rich resources for many organisms including microbes and insects. Microbes were initially thought of solely as nutrient recyclers[60]. Janzen[61] suspected microbes compete with other consumers for these resources, and they produce compounds, such as toxins, affecting resource "appeal" thus

reducing competition. This principle was demonstrated by Burkepile et al. [8]. Microbes colonizing fish carrion in tidal estuaries competed with other consumers for these resources [8]. These microbes released noxious chemicals that deterred consumption of the fish remains by higher level consumers, such as crustaceans[8]. Removing the microbes resulted in the carrion being attractive to crustaceans for significantly more time[8].

***Chrysomya rufifacies* is a dominant decomposer in Texas.** Blow flies are the dominate decomposers of human remains. *Cochliomyia macellaria*, the secondary screwworm, is native to the U.S. and was the most common colonizer of animal carcasses in the southern U.S. and tropics[62] prior to the introduction of *C. rufifacies*[63] from tropical Australia and the Orient in 1978[64,65]. *Chrysomya rufifacies* larvae compete for resources and are predaceous on *C. macellaria* and *L. sericata* larvae[63,66]. In most instances, *C. macellaria* larvae disperse from a carcass when *C. rufifacies* colonizes the same resource thus increasing risk for mortality [63] and impacting (positive or negative) its population dynamics[67]. As noted elsewhere, we have evidence that this species uses microbial cues to colonize a body.

We propose to study the manipulation of bacterial populations by larvae of *C. rufifacies* and *L. sericata* on ephemeral resources. **We predict** such microbial-insect interactions can be modeled resulting in a method for quantifying the PMI well beyond the initial 72 h regardless if insects are present or not. Our data indicate volatiles from bacterial communities on decomposing tissue attract or repel blow flies competing for these resources[22,68]. Furthermore, bacteria associated with immature blow flies also serves to attract intra- and inter-specific adults. We will conduct a series of field and laboratory experiments to quantify bacterial succession and resulting interactions with blow flies that colonize these resources.

STATEMENT OF HYPOTHESES AND RATIONALE

Objective 1. Microbial-arthropod successional interactions for estimating the PMI of a corpse. The structural and functional changes in microbial communities on decaying human carcasses and decaying human tissue in laboratory conditions will be described. We will identify specific microbial communities and their associated metabolic processes that influence the initial attraction, arrival sequence, and interactions of blow flies to a decomposing resource.

Objective 1 Hypothesis: Microbial function and structure can be used to estimate the PMI of human remains.

Objective 1 Rationale. Our current research indicates microbial communities on pig remains proceed through a succession process. This proposal is focused on the identification of colonizing bacterial species, determining the predictability of their succession, and determining the use of this information in estimating the PMI of human remains. This study will examine the effects of specific bacteria on blow fly species utilization of carcass tissue. Microbiome information from this research will allow us to identify trends related to colonization and identify important bacterial species. All sequence data will be made available to the scientific community through the NCBI databases. Research and data analyses will be published in peer-

reviewed scientific journals. We will also compare these data to those generated from our pig studies to further validate the pig as an acceptable model in place of human remains.

Objective 2. Conduct lab validation studies to quantify variation association with microbial succession on human tissue.

Objective 2 Hypothesis: Microbes influence arthropod attraction, colonization and development on human tissue.

Objective 2 Rationale. The two goals listed in objective 2 examine interactions between the microbes and blow flies in greater detail. Blow flies from the *Lucilia* genus are primary colonizers of decomposing remains[33] and engage in facultative myiasis [69]. This behavior can have devastating economic effects, as demonstrated by fly-strike on sheep by *L. cuprina* [70,71], but can also be beneficial as demonstrated by the use of *L. sericata* in maggot therapy, which uses the larvae of this and similar species to efficiently debride necrotic wounds [24].

Interestingly, microbes have been shown to play an important role in *Lucilia* biology. Fly-strike by *L. cuprina* results from female oviposition on feces found in the wool of sheep. The mechanism of this attraction is dependent (in part) on volatiles released by *Pseudomonas* species metabolizing wool (Table 1). Furthermore, the use of *L. sericata* in maggot therapy has led to numerous microbiological studies of this species, demonstrating that the excretions and secretions (ES) of *L. sericata* have antimicrobial properties[72,73] and can affect the formation of biofilms[74]. Further, there is specificity to its potential to kill microorganisms[75]. Three genera *L. sericata* does not kill are *Pseudomonas*, *Proteus*, and *Providencia*, which attract blow flies and have been found in association with *Lucilia* colonies (Tarone, unpublished data). These results are suggestive of potential mutualisms between *Lucilia* and certain bacteria (like *Proteus*), meaning the presence of flies may kill some bacteria in a community while promoting the growth of others[23]. Given the importance of this genus in forensic entomology and its demonstrated potential to alter microbial communities, it is reasonable to predict that microbe-insect interactions are an important factor that needs to be considered when predicting the PMI with microbes and blow flies.

II. METHODS

Objective 1 (c) Standard Operating Procedure for Analyzing and Interpreting Microbes: The methods previously described will be formulated into a written standard operating procedure that will be tested in Objective 3.

Objective 2. Conduct lab validation studies to quantify variation association with microbial succession on human tissue.

2 (a) Abiotic factors affecting select microbes: Controlled observations of microbial communities associated with decomposing human remains will be done to clarify results obtained from field observations. Using a select set of microbes identified in 1 (b) a series of experiments will be conducted to determine the effects of humidity (25% and 75% RH) and

temperature (20°C and 34°C) on the proliferation of our species of interest on human tissue. As an example, we find *Proteus* species commonly associated with *L. sericata*, which are similar to strains implicated in the elimination of other microbes on a wound colonized by flies[23]. The growth of four key strains will be evaluated on human tissue over time in three replicates of each of the four possible combinations of humidity and temperature to determine abiotic influences on key players in the microbial communities observed in the first objective. Bacterial strains will be chosen based on their potential to be a temporal marker of human decomposition, high abundance, and prevalence across all cadavers studied in Objective 1. Human tissue will be seeded with a standardized number of bacterial cells, which will be determined experimentally for each species to ensure that the microbes do not overpopulate the resource during the duration of the experiments, in each of the environmental combinations. The standardized number of cells will represent an ecologically relevant density. Then five samples will be taken from five sites on the tissue and pooled over the course of 72 h. Three tissue samples will be used as replicates. Quantitative PCR will be conducted on each sample to determine the concentration of bacteria in a standard volume removed from the tissue. CFUs will be determined and the $\log_{10}$ change after exposure calculated. In addition, samples collected for quantitative PCR will also be applied to Biolog plates (as described above) to observe the effects of these key microbes on community function and how the abiotic factors studied will affect those functions.

Microbe communities that the study bacteria will be cultured with will be evaluated to determine effects of variation in microbe community on their growth. Samples will be taken from the tissue at the beginning and end of each experiment to determine the effects of 1) the initial community structure on growth of the four target species and 2) the effect of the study species on ultimate community structure. Pyrosequencing methods will be done as noted above.

2 (b) Biotic factors affecting select microbes: The effects of *C. rufifacies* and *L. sericata* (eggs, larvae, adults) on bacteria communities associated with decomposing human tissue will be quantified. Methods for raising sterile larvae will be adapted from Sherman and Tran [24]. The Tarone and Tomberlin laboratories are currently capable of rearing sterile flies, which do not demonstrate microbial growth after 72 h of incubation of larval tissue in sterile medium. A single clutch of eggs will be agitated in 1% sodium sulphite for 10 min and surface sterilized per Ahmad et al.[25]. Eggs will then be rinsed with saline, placed in sterile containers, and fed previously prepared sterile liver media. Pupae will be removed, sterilized using methods previously described, placed in sterile petri dishes in a growth chamber. Emergent flies will be placed in sterile enclosures, provided sterile food *ad libitum*. Eggs will be collected using the sterile liver:agar mixture. Resulting eggs will be used in the following experiments after surface sterilization.

Fly Colonies. Approximately 1,000 adult *L. sericata* and *C. rufifacies* respectively will be sampled from decomposing animal remains located in College Station, Texas to get a sufficient genetic sample. Cages will be maintained in the laboratory at 27°C and 60% RH. Water with 50% sugar concentration will be provided. Cages, sugar water, and consumables will be autoclaved prior to use.

Experiment 1. Parallel comparison of bacterial communities growing on human tissue when exposed to L. sericata *and* C. rufifacies*.* The four aerobic bacteria identified above will be

examined in this study. All human tissue used in the experiments described in Objective 2a will be split between 2a and 2b, which will allow direct comparisons between experiments. For each bacterium/blow fly combination, the bacterial species will be spiked onto the tissue (Table 1) and raised in the temperature and humidity condition from Objective 2a that is most relevant to typical environmental conditions near College Station, TX (i.e., the high humidity, high temperature condition described in Objective 2a). All analyses done in Objective 2a will also be conducted in this experiment, to enable a comparison of microbe communities in the absence of blow flies to those in their presence.

Experiment 2: Significance of bacteria-larval interactions on larval development. Entomological evidence is a potential data source informative of a PMI. It is entirely possible that different bacteria associated with human remains may alter the development rates of blow fly species. Accordingly, knowledge of microbial influences on blow fly development will be useful in identifying factors that affect error in entomologically based PMI estimates. Effects of direct contact of bacteria on fly development will be assessed.

Growth curves will be conducted on each bacterial species to determine the optimal starting concentration and sampling frequency, so as not to overgrow during the three day experiment. Individual bacteria (A&B represent two bacteria species from *L. sericata* and C&D represent bacteria species from *C. rufifacies*) will be plated on appropriate agar at two different concentrations, appropriate to their growth rate (e.g. 10^2, 10^4 cfu/ml) and incubated at 34^{o}C. Three replicates of each treatment previously listed will be conducted for a total of 144 plates per fly species. Sterility of eggs will be confirmed prior to treatment with experimental bacteria. For each treatment, at least fifteen eggs from the identified blow fly species will be obtained from colonies previously described, surfaced sterilized (see above) and placed on each replicate. Per Ahmad et al. [25], plates will be placed in a growth chamber set at 34°C and monitored daily for larval mortality and pupation. For the flies, immature survivorship, pupal weight, and development duration will be recorded. Emergent adult weight will also be recorded. A sample will be taken at each stage of development (eggs, larvae, pupae and adult flies) for determination of bacterial concentrations by serial dilution.

Data Analysis: Geometric mean, 95% confidence interval, of bacterial colony forming units will be determined. ANOVA will be used to analyze CFU, larval survival and fly species interaction[26]. Least Significant Difference (LSD) test was used following a significant ($P < 0.05$) F test to separate means[26]. All percent data will be arcsine transformed prior to analysis.

III. RESULTS

***Note:** Publications generated by use of personnel funded by this NIJ project to conduct bioinformatics analyses on datasets previously or concurrently collected on other projects related to the concepts in this grant. These smaller "training" datasets allowed the development of a bioinformatics and statistical pipeline for the much larger and more complex dataset generated by the work in this grant.

Accomplishment #1 Objective 1.

1. ***Developing and testing field sampling protocols to describe bacterial community structure and microbial community function on decomposing vertebrate remains):** We have conducted several experiments demonstrating we can effectively sample microbial communities from multiple locations on decomposing vertebrate remains, including swine as surrogates of human remains. These studies also, for the first time, associated the bacterial communities of decomposing remains with colonizing insect communities. We have two papers **(Appendices 1&2)** in review and one that is in press that demonstrate our success in sampling the communities and processing and analyzing them using 454 pyrosequencing and metabolic profiles.

2. **Three field trials were conducted.** Human remains arriving to the facility simultaneously as a pair was a major concern with our research, as temporal variation could result in significant differences between the microbial communities associated with the remains examined. We were fortunate to have access to human remains as pairs (arriving within 10 days of each other) during November 2011, May 2012 and November 2012. In cases where remains did not arrive on the same day, the first set of remains was stored in a cooler at 4°C until the second set of remains arrived. We were able to examine microbial community function and structure in relation to insect activity on human remains, which was the main emphasis of objective 1.

3. **Pyrosequencing.** We were able to collect samples from all remains, isolate and amplify bacterial DNA and have the 16s rRNA region sequenced for bacterial identification. We are currently analyzing these data in preparation for publication. Preliminary analyses of the combined data from first trial suggets that insect plays a significant role in microbial succession of human cadavers, because microbial community structure of insect access cadaver (ACC) was significantly different than insect exclusion cadaver (EXC) in AMOVA analyses ($P < 0.0002$) (Figure 18). Among all sequences, more than 80% belonged to phyla Proteobacteria, Firmicutes, and Actinobacteria in decreasing order (Figure 19). In general, relative sequence abundance of proteobacteria increased with time after placement of the cadaver, whereas relative sequence abundances of Firmicutes and Bacteroidetes decreased with time, and this pattern was almost similar in both ACC and EXC cadavers (Figure 19). Relative abundance of sequences classified at genus level however was very different in ACC and EXC cadavers, and this difference gradually incraesed with time after placement of cadavers (Figure 20). Bacterial diversity associated with soil samples were significantly different compared to swab samples, and same was true between swab samples collected from different body sites (Figure 21). However, bacterial diversity of soil collected from directly under the body was not significantly different than bacterial diversity of soil 1 m away from the cadavers (AMOVA $P = 0.0748$), which suggests that soil bacteria are not very useful for short term PMI estimation, but it may provide useful information for long term PMI estimation (Figure 21). Significant difference in bactrial structure between swab samples collected at different time points from ACC cadavers (AMOVA $P < 0.0002$; Figure 22), suggests that under normal conditions (with insect access) bacterial succession can be utilized as a microbial clock for estimation of PMI. This is mainly because relative sequence abunadnce of many indicator genera either increases (*Ignatzschineria, Acinetobacter*, *Wohlfahrtiimonas*) or decraeses (*Anaerococcus*, *Finegoldia*) with time (Figure 23).

4. ***Pyrosequencing support research (The potential use of bacterial community succession in forensics as described by high throughput metagenomic sequencing).** Decomposition studies of vertebrate remains primarily focus on data that can be seen with the naked eye, such as arthropod or vertebrate scavenger activity, with little regard for what might be occurring with the microorganism community. Here we discuss the necrobiome, or community of organisms associated with the decomposition of remains, specifically, the 'epinecrotic' bacterial community succession throughout decomposition of vertebrate carrion. Pyrosequencing was used to 1) detect and identify bacterial community abundance patterns that described discrete time points of the decomposition process, and 2) identify bacterial taxa important for estimating physiological time, a time-temperature metric that is often commensurate with minimum post-mortem interval estimates, via thermal summation models. There were significant bacterial community structure differences in taxon richness and relative abundance patterns through the decomposition process at both phylum and family taxonomic classification levels. We found a significant negative linear relationship for overall phylum and family taxon richness as decomposition progressed. Additionally, we developed a statistical model using high throughput sequencing data of epinecrotic bacterial communities on vertebrate remains that explained 94.4% of the time since placement of remains in the field, which was within 2-3 hours of death. These bacteria taxa are potentially useful for estimating the minimum post-mortem interval. Lastly, we provide a new framework and standard operating procedure of how this novel approach of using high throughput metagenomic sequencing has remarkable potential as a new forensic tool. Documenting and identifying differences in bacterial communities is key to advancing knowledge of the carrion necrobiome and its applicability in forensic science. (*Published* in International Journal of Legal Medicine: (**Appendices 1&3**). The International Journal of Legal Medicine also provides a standard operating procedure for collecting, analyzing and interpreting microbial community data associated with estimating the PMI of the remains, satisfying part of Objective 1(c).

5. ***Biolog EcoPlates™.** As with the pyrosequencing, we were able to analyze samples for functional shifts in microbial communities associated with the human remains throughout the decomposition process. These data will be analyzed in parallel with the pyrosequencing data to determine if structural and functional shifts are related and can be used individually or in tandem to make estimates of the minimum PMI of decomposing remains. An initial assessment of this these relationships between structure and function have been included in a paper accepted for publication in PLoS ONE (see below). In that paper it is demonstrated that as microbial metabolic profiles change during decomposition, so do the taxa at least at the phylum level.

6. ***Biolog EcoPlates™ support research (Microbial Community Functional Change During Vertebrate Carrion Decomposition):** Microorganisms play a critical role in the decomposition of organic matter, which contributes to energy and nutrient transformation in every ecosystem. Yet, little is known about the functional activity of epinecrotic microbial communities associated with carrion. The objective of this study was to provide an initial description of carrion associated microbial community functional

activity described by differential carbon source use throughout decomposition over seasons, between years and when microbial communities were isolated from eukaryotic colonizers (e.g., necrophagous insects). Additionally, microbial communities were identified at phylum level using high throughput sequencing during a single study. We hypothesized that carrion microbial community functional profiles would correspond with stage of decomposition, and that functional change would depend on season, year and presence of necrophagous insects. Biolog EcoPlates™ were used to measure the variation in epinecrotic microbial community by the differential use of 29 carbon sources as vertebrate carrion decomposed. Pyrosequencing was used to identify the microbial communities throughout carrion decomposition. Overall, microbial functional activity increased throughout decomposition in spring, summer and winter while it decreased in autumn. Additionally, microbial functional activity was higher in 2011 when eukaryotic colonizer effects were tested. There were inconsistent trends in microbial function from communities that were isolated from necrophagous insects between 2010 and 2011. These data are important in understanding the influence of microbial communities on an essential ecosystem function, carrion decomposition. Further, our results contribute to the growing knowledge base of microbial communities associated with ephemeral resource pulses and their influence on decomposition processes (**Appendix 2**).

7. **Human vs. swine (pyrosequencing and Biolog EcoPlates™).** In an overlapping study, for each of our three human trials, we conducted a simultaneous comparison study to swine carrion (funded separately by these investigators). The objective was to research statistically if swine are an appropriate model for conducting forensic microbiology research. Swine remains are often used as a surrogate for human remains when conducting forensic entomology-based research. These data have been collected and are currently being analyzed for comparison to the human remains data.

8. **Differences among human remains MMCPs in all field trials (H1-H6):** There were significant differences among trials (PERMANOVA: $P < 0.001$); no significant differences between treatment or sex, and a significant interaction ($P = 0.008$); no significant difference between treatment or among sampling days, and no significant interaction; and no significant difference between sex or among sampling days, and no significant interaction. A two-dimensional NMDS ordination (stress = 0.189, $R^2 = 0.841$) described the variation in normalized MMCPs among field trails (Figure 3). Because there were significant differences in MMCPs among trials, each trial was subsequently analyzed individually.

   a. ***Differences between human remains MMCPs in field trial 1 (H1 & H2)*****:** There were significantly different MMCPs between treatments (PERMANOVA: P = 0.015). A two-dimensional NMDS ordination (stress = 0.094, $R^2 = 0.970$) described the variation in normalized carcass MMCPs in field trial 1 (Figure 4).

   a1. Differences in ACC human remains (H1) MMCPs during field trial 1: There were no significant changes in MMCPs among sampling days, sampling areas (buccal, skin, anal) and no significant interaction. A two-dimensional NMDS

ordination (stress = 0.077, R^2 = 0.975) described the variation in normalized carcass MMCPs in field trial 1 (Figure 5).

a2. Differences in EXC human remains (H2) MMCPs during field trial 1: There were no significant changes in MMCPs among sampling days, sampling area and no significant interaction. However, there was a significant difference among sampling areas without account for day of decomposition (PERMANOVA: P < 0.001). A two-dimensional NMDS ordination (stress = 0.046, R^2 = 0.995) described the variation in normalized carcass MMCPs in field trial 1 (Figure 6).

b. Differences between human remains MMCPs in field trial 2 (H3 & H4): There were no significant differences in MMCPs between treatments, sampling day, and no significant interaction. Furthermore, there were no significant differences between sampling area, sampling day, and no significant interaction. A two-dimensional NMDS ordination (stress = 0.148, R^2= 0.902) described the variation in normalized carcass MMCPs in field trial 2 (Figure 7).

c. Differences between human remains MMCPs in field trial 3 (H5 & H6): There were no significant differences between treatments, sampling day, and no significant interaction. There were also no significant differences between sampling area, sampling day, and no significant interaction. A two-dimensional NMDS ordination (stress = 0.216, R^2= 0.795) described the variation in normalized carcass MMCPs in field trial 3 (Figure 8).

d. Differences between ACC human remains (H1, H3, H5) and swine carcasses (P1-P3) MMCPs in all field trials: There were significant differences (P=0.006) among trials, no significant differences between remain types (human vs. swine) and no significant interaction. A two-dimensional NMDS ordination (stress = 0.189, R^2= 0.856) described the variation in normalized carcass MMCPs among all field trials (Figure 9). Because there were significant differences in MMCPs among trials, each trial was subsequently analyzed individually.

e. Differences between ACC human (H1) and swine (P1) remains MMCPs in field trial 1: There were no significant differences between remains species, among sampling days and no significant interaction. There were no significant differences among sampling areas or among sampling days, but there was a weakly significant interaction (P = 0.030). A two-dimensional NMDS ordination (stress = 0.113, R^2 = 0.947) described the variation in normalized carcass MMCPs in field trial 1 (Figure 10).

f. Differences between ACC human (H3) and swine (P2) remains MMCPs in field trial 2: There were no significant differences between remains species, among sampling days and no significant interaction. There were no significant differences among sampling areas, among sampling days and no significant interaction. A two-dimensional NMDS ordination (stress = 0.159, R^2 = 0.886) described the variation in normalized carcass MMCPs in field trial 1 (Figure 11).

g. Differences between ACC human (H5) and swine (P3) remains MMCPs in field trial 3: There was a significant difference ($P = 0.004$) in MMCPs between remain species, but no significant differences among sampling days and no significant interaction. A two-dimensional NMDS ordination (stress = 0.170, $R^2 = 0.901$) described the variation in normalized carcass MMCPs in field trial 3 (Figure 12).

h. Differences in ACC human remains (H5) MMCPs during field trial 3: There were no significant differences among sampling days or sampling areas (buccal, skin, anal) and no significant interaction. A two-dimensional NMDS ordination (stress = 0.121, $R^2 = 0.945$) described the variation in normalized carcass MMCPs in field trial 3 (Figure 13).

h1. Differences in swine remains (P3) MMCPs during field trial 3: There were no significant differences among sampling days or sampling areas (buccal, skin, anal) and no significant interaction. A two-dimensional NMDS ordination (stress = 0.134, $R^2 = 0.929$) described the variation in normalized carcass MMCPs in field trial 3 (Figure 14).

i. Differences among swine carcasses (P1-P3) MMCPs in all field trials: There were significant differences ($P < 0.0001$) among trials when the swine carcasses were compared. A two-dimensional NMDS ordination (stress = 0.169, $R^2 = 0.885$) described the variation in normalized carcass MMCPs among all field trials (Figure 15).

j. Differences among swine carcasses (P1) MMCPs in Trial 1: There were no significant differences among sampling days, sampling area (buccal, skin, anal) and no significant interaction. A two-dimensional NMDS ordination (stress = 0.078, $R^2 = 0.973$) described the variation in normalized carcass MMCPs in field trial 1 (Figure 16).

k. Differences among swine carcasses (P2) MMCPs in Trial 2: There were no significant differences among sampling days, sampling area (buccal, skin, anal) and no significant interaction. A two-dimensional NMDS ordination (stress = 0.154, $R^2 = 0.883$) described the variation in normalized carcass MMCPs in field trial 2 (Figure 17).

l. Differences among swine carcasses (P3) MMCPs in Trial 3: Please see results in section h1.

9. ***Volatile organic compound production.** We were able to collect volatile organic compounds (VOCs) emitted from the human remains in our experiments. We wanted to demonstrate a relationship between microbial activity in association with abiotic and biotic variables (objective two). We also collected VOCs from the swine carcasses. The data from human remains in trials 1 and 3 did not identify any meaningful variation between the treatments groups (ACC and EXC) using principle components analysis (PCA); the first component extracted less than 50% of the variation within the data set

and distinct clustering of points was not achieved. A number of compounds from a variety of chemical classes were identified from the samples; however, no significant trends were apparent in trials one or three. During trial 3, the number of collection days was extended from four to six and the human remains were exposed to slightly warmer temperatures (average 18.9°C vs. 15.2°C). We identified decomposition VOCs such as polysulfides (DMDS, DMTS, DMQS), phenol and pentanal in the decomposition headspace. These VOCs were also detected from the swine carcass from trial 3. Principle components analysis of VOCs from the human remains from trial 2 (May 2012) were significantly different from the VOCs collected from the swine carcass. Furthermore, the insect inclusion remains were differentiated from those collected from the human in the exclusion treatment during the later experimental days. However, there was no differentiation of the later decomposition stages based on headspace chemical composition in the EXC human remains. The swine carcass and ACC human remains in trail 2 were characterized by alcohols and sulfides on experimental days 4 and 5. Although the PC loadings for the alcohols and sulfides were lower (component 1: sulfide -0.3567, alcohol -0.2006; component 2: sulfide -0.6838, alcohol -0.3050), there was a distinct separation of these data points in the lower left quadrant. These compounds were detected earlier from the swine carcass, with DMDS first detected on experimental day 2 (ADD 54.4) while detected on experimental day 3 (ADD 79.5) for the human ACC remains. Overall, the chemical profiles from the human ACC remains appeared to lag behind that of the swine carcass, which had a greater diversity and increased levels of compounds earlier in the decomposition process. (**Appendix 4**)

10. ***Blow fly cuticular hydrocarbon profiles:** Because cuticular hydrocarbons of blow flies are thought to play a role in attracting or repelling mates, these volatile signatures have the potential to interact with VOCs being emitted from a decomposing corpse, thereby affecting blow fly response to the corpse and possibly subsequent colonization. We identified 25 and 26 hydrocarbons associated with lab-reared *C. macellaria* and *Ch. rufifacies*, respectively, with 10 compounds shared between species. Nonmetric multidimensional scaling analysis and permutational multivariate analysis of variance were used to analyze the profiles, and detected significant profile differences ($P < 0.001$) between sampling days for each species. It was found that adult *C. macellaria* specimens associated with decomposing swine carcasses had subtle changes in their hydrocarbon profiles when collected on the first two days of decomposition. However, *C. rufifacies* demonstrated more variable profiles within the first three sampling days. Our lab-reared specimens demonstrated unique chemical 'fingerprint profiles' most likely due to the exposure to a constant temperature (~ 26^{o}C) while the field-collected specimens were exposed to fluctuating temperatures ranging from ~ 18 - 39^{o}C. Additional field collected specimens need to be collected over a greater temporal scale (i.e., greater than 3 days) and in different abiotic conditions as it is well know that temperatures will affect the hydrocarbon structures in insect cuticle.

11. ***The blow fly internal microbiome:** As part of this objective, pyrosequencing was used to understand bacterial succession during corpse decomposition. In order to better understand how blow fly contact and colonization affects this succession by introducing exogenous taxa to the epinecrotic communities, the internal microbiome of adult blow fly

species were evaluated using pyrosequencing. Different species were collected in the field using bait traps from several populations representing different ecoregions of the Appalachian Mountains. Several populations were collected and analyzed for their internal microbiome with the objective to understand what taxa are common among specific species even in different ecoregions compared to those taxa that are unique to specific populations or ecoregions, and test if there were sex specific bacterial communities with or across ecoregions. So far the following six phyla make up > 99% of the bacterial communities from *Phormia regina* adults collected: Actinobacteria, Bacteroidetes, Chloroflexi, Firmicutes, Tenericutes and Proteobacteria. Using permutational multivariate analysis of variance (PERMANOVA), there were significant differences between sex ($P = 0.002$), no significant differences among ecoregions and no significant interaction. There were no bacteria phyla differences between sex, but the females were significantly higher than males in Shannon-Wiener diversity, Simpson's diversity and evenness across ecoregions. This provides one of the first data sets that will allow for comparisons with bacteria that are commonly found on corpses and how they change during decomposition. Additional studies are being developed to capture adult and larval blow flies associated with specific decomposing swine carcasses or human remains where the epinecrotic communities will be matched with the adult and larval microbiomes. This information will identify which bacterial taxa are already associated with decomposition and those that are brought in by the blow flies (exogenous taxa) and modified by larval activity.

Accomplishment #2: Objective 2.

1. ***Sterilization techniques:** We developed methods for sterilizing fly eggs that removed the associated microbes. The sterile eggs were then used to conduct controlled experiments examining the influence of bacteria on the behavior and ecology of blow flies associated with human decomposition. We have developed a manuscript for this project and will be submitting it for publication shortly. (**Appendix 5**).

2. ***Blow fly egg microbes regulate attraction of blow fly adults:** We have conducted a controlled laboratory experiment demonstrating the microbes associated with blow fly eggs (*Chrysomya rufifacies* and *Cochliomyia macellaria*) regulate blow fly attraction and possibly colonization. We also conducted a preliminary experiment identifying the egg-associated bacteria from each fly species. We have a manuscript prepared for this project and will be submitting it shortly. (**Appendix 5**).

3. ***Bacterial succession through life stages of a fly:** We published one manuscript that examined microbial succession through the black soldier fly (BSF), a colonizer of carrion. This paper partially demonstrated that bacteria associated with BSF were vertically and horizontally transmitted. We demonstrated the bacterial populations varied from stage to stage, which indicated that the insect regulates bacterial community assembly. Using 454 sequencing, we surveyed bacteria associated with successive life stages of BSF reared on plant material. Bacterial diversity classified (99.8%) across all life stages spanned six bacterial phyla with ≥80% bootstrap support. Bacteroidetes and Proteobacteria were the most dominant phyla and accounting for 66% of the fauna identified. Many of the bacteria taxa identified would go undetected without 454 sequencing due to their inability to be cultured (**Appendix 6).**

4. ***Bacteria from carrion arthropods and associated concentrations regulate attraction and colonization of carrion:** We published a manuscript detailing the role of bacteria from different arthropod species associated with carrion on the attraction of black soldier flies (BSFs) to decomposing resources. There can be substantial negative consequences for insects colonizing a resource in the presence of competitors. We hypothesized that bacteria associated with an oviposition resource and the insect eggs deposited on that resource serve as a mechanism regulating subsequent insect attraction, colonization and potentially insect succession patterns. We isolated and identified bacterial taxa associated with insects (e.g., *C. macellaria*, *C. rufifacies*, and *A. diaperinus*) associated with vertebrate carrion and used these bacteria to measure their influence on the oviposition preference of adult BSFs, which utilizes animal carcasses and is an important species in waste management and forensics. We demonstrated that utilization of a mixture of bacteria, rather than a single species, differentially influenced behavioral responses of BSFs, as did bacterial concentration and the fly species from which the bacteria originated. These studies provide insight into interkingdom interactions commonly occurring during decomposition, but not commonly studied **(Appendix 7)**.

5. ***Volatile production from mutant bacteria.** We are describing the VOCs associated with key bacterial species known to occur on decomposing remains. The goal is to link the VOCs produced by these bacteria with arthropod attraction. Furthermore, we aim to link VOC production with microbial community structure. If successful, VOCs could indicate community structure as related to the amount of time to have passed since death **(Cuttiford, Ph.D. dissertation to be awarded 2016).** This information will also be compared to the flow fly cuticular hydrocarbon profiles being evaluated for Objective 1.

6. ***Similarity and differences in bacterial communities through life stages of three flies associated with carrion:** Although there are several studies on bacteria living symbiotically with insects, studies on bacteria associated with flies of forensic importance are lacking and limited to those that can be cultured. Using 454 sequencing of 16S rDNA, we examined bacterial diversity associated with *Hermatia illucens* (black soldier fly), *Lucilia sericata* and *L. cuprina*. Total 4852, 16557, and 15110 sequences were obtained from *H. illucens*, *L. sericata*, and *L. cuprina*, respectively. These sequences were classified into 7 phyla, 15 classes, 25 orders, 67 families and 121 genera. Bacterial diversity was higher and significantly different in BSF than in either *Lucilia* species. Bacteroidetes (42%), Firmicutes (50%), and Proteobacteria (84%) were the most dominant phyla associated with *H. illucens*, *L. sericata* and *L. cuprina*, respectively. Among all genera that were present at 2% or higher level, only *Providencia* was shared among all fly species included in this study. *Lucilia sericata* and *L. cuprina* shared the most number of bacterial genera and each fly species had unique taxa that were either absent in other fly species or present in a negligible quantity. Understanding the relationship between bacteria and their associated fly hosts could provide insight to their ecological relevance in regards to resource location, allocation and utilization. These relationships could explain the larval development in forensic studies and consequently increase precision of the estimated time of colonization of human remains.

7. ***Microbes associated with fly larvae regulate attraction and colonization by blow flies:** We conducted an experiment demonstrating the microbes associated with third instar *Chrysomya rufifacies* larvae regulated attraction and colonization of a decomposing resource by a competing fly, *Cochliomyia macellaria*. We are currently developing a manuscript for this project **(Appendix 8).** This information will also be compared to the adult internal microbiome from field captured flies (Objective 1) to better identify the potential for blow flies to bring in exogenous bacteria to a corpse during decomposition.

8. ***Key microbial species associated with decomposing human remains:** Genetic differences Insect-microbe interactions are well documented and are the basis for this research project. *Proteus* and *Providencia* species are commonly associated with blow flies and it is also known that *Proteus* and *Providencia* spp. will attract blow flies. Both bacterial genera were identified from the remains in these studies. The blow flies of genus *Lucilia*, (Diptera: Calliphoridae) can be primary colonizers during decomposition and are important to forensic science as data useful for estimating a postmortem interval. Clearly, microbes and their associated metabolites can influence blow fly behavior, with potential repercussions for forensic applications. Previously these investigators had collected bacteria of these genera from blow flies and these wild type bacteria are being sequenced by next generation sequencing techniques for comparison against sequences from clinical isolates. Comparison results will give us a better understanding of genetic differences, and hypotheses on possible functional differences or responses to environmental influences and possibly genes important to the interactions between blow flies and microbes that may influence their behavioral choices, such as feed or oviposition.

9. ***Microbial volatiles linked to nutrition of carrion source impact fly attraction.** Dimethyl Disulfide (DMDS) is a known VOC and one of the prominent sulfur VOCs emitted by decaying vertebrate remains. *Proteus* and *Providencia* species are commonly associated with blow fly larvae and adults; these bacteria were able to degrade methionine and produced DMDS therefore linking bacteria to fly colonization through VOC production. In vertebrate carrion ecology, *L. sericata*, are typically the first arthropods to colonize carrion. Consequently, these insects have been selected to have highly sensitive olfactory systems to detect low levels of VOCs indicative of the presence of carrion sources. It is suspected that DMDS might be a key VOC utilized by insects to direct/locate food or oviposition sites. The responses of 7-9 d old *L. sericata* adults to DMDS at different doses were examined in a dual-choice olfactometer. Logistic regression was used to analyze this behavioral data taking into account dose, sex and ovarian development on the response of the flies. Behavioral responses to DMDS were variable by different doses. DMDS was repulsive for gravid females while marginally attractive for males at 0.005 µg. DMDS was also marginal attractive to males at 10 µg. Sex was significance in explaining the choice fly made in the bioassay in response to DMDS at dose 0.005 µg and 0.05 µg. These data presented in the current study demonstrate a relationship between sex and physiological state with regards to attraction to a VOC that occurs during decomposition. Understanding this relationship could provide insight to the mechanisms regulating subsequent colonization and arthropod

succession and further clarifying previous work with this compound regarding the timing of attraction of different phenotypes (male, non-gravid and gravid females) to remains. **(Liu Ph.D. Dissertation to be completed August 2014).** This information will also be compared to the flow fly cuticular hydrocarbon profiles being evaluated for Objective 1.

10. **Bacterial quorum sensing regulates arthropod behavior.** As with Objective 2, we were interested in how bacteria regulate arthropod behavior- specifically as related to attraction and colonization of human remains. We determined in our study that quorum sensing by bacteria serves as one of these mechanisms. Quorum sensing molecules used to regulate microbial behavior were found to influence blow fly attraction and colonization of a resource. This research could lead to novel methods for monitoring specific chemicals produced by bacteria and predicting a min-PMI of human remains **(Appendix 9)**.

11. ***Physiological response of flies to volatiles.** We currently have one PhD student conducting research examining the physiological responses of *L. sericata* to DMDS, which is a known VOC consistently produced from decomposing human remains. Our goal is to determine if adult flies collected from a death scene can be examined for physiological shifts allowing for an estimation of the time of death in the absence of arthropod colonization (e.g., laravae). We currently have data suggesting that adult life span and defecation rate are altered when adult flies are exposed to DMSD. Furthermore, we have determined that the responses of *L. sericata* were related to the physiological state (e.g., male, non-gravid or gravid female). We are attempting to develop protocols that would allow investigators and forensic entomologists to successfully use this information in an investigation **(Liu Ph.D. Dissertation to be completed August 2014).** This information will also be compared to the flow fly cuticular hydrocarbon profiles being evaluated for Objective 1.

12. ***Fly larval response to essential amino acids.** Carrion associated microbes and arthropods, such as blow flies, fill critical roles in the degradation of human remains. It is known that human remains are heterogenous resource patches with variable nutritional quality. These nutrients are important in regulating microbe and arthropod population dynamics such as succession patterns. At the most fundamental level, the micronutrients (specifically the essential amino acids) regulate the success and phenotypic responses of microbes and insects. Therefore, we have developed a method allowing us to shift the presence and concentration of these essential amino acids and measure the corresponding phenotypic responses of flies. Preliminary data indicated the presence/absence of essential amino acids regulated feeding preferences of blow fly larvae. We have also linked the presence/absence of these amino acids with VOCs that are emitted from the remains. Our hypothesis is the health of an individual and corresponding nutritional makeup preceding death, play an important role in regulating arthropod attraction and colonization of human remains. Furthermore, these nutrients regulate microbial succession patterns **(Liu Ph.D. Dissertation to be completed August 2014)**. This information will also be compared to the flow fly cuticular hydrocarbon profiles being evaluated for Objective 1.

13. ***Development of an artificial sterile diet.** The use of human tissue was prohibitive for this study due to an inability to develop proper sterilization techniques of the material prior to experimental use. The goal of this study is to develop a sterile diet allowing for controlled laboratory experiments examining the impact of nutrients on the development of blow flies and microbes. **(Zheng Ph.D. Dissertation to be completed August 2016).**

Accomplishment #3: Undergraduate student training.

1. **Riddle, B., and J.K. Tomberlin, 2013.** Mr. Riddle was majoring in Forensic & Investigative Sciences at Texas A&M University. Barrett examined the role of nutrition in interkingdom signaling between bacteria and *Lucilia sericata* (Diptera: Calliphoridae). Quorum sensing molecules that bacteria use to communicate are partially derived from the breakdown of several amino acids. He is manipulating the presence of these amino acids in an artificial diet in order to determine the behavior of the fly larva as well as the production of volatile organic compounds that serve as important mechanisms regulating adult fly attraction and colonization.

2. **Evers, K., and J.K. Tomberlin. 2013.** Ms. Evers was a senior Forensic and Investigative Sciences major and is minoring in Chemistry at Texas A&M University. Her research examined the effects of deterring insect colonization of swine carrion on associated microbial function. This information could help to determine a more accurate minimum postmortem interval (m-PMI) of a body by elucidating the pre-colonization interval which is the time period before insects have arrived and colonize the corpse.

3. **Henger, N., and J.K. Tomberlin. 2013.** Ms. Hneger was an undergraduate student at the University of Dayton. She worked with Ms. Wenqi Lui and Dr. Singh Baneshwar and was mentored by Dr. Jeff Tomberlin. Nichole examined the role quorum sensing-related volatiles play in eliciting gene level responses and resulting downstream behaviors by the blow fly *Lucilia sericata*. Her research sheds light on the interkingdom interactions between bacteria, its host and bacteria on host resources. Nichole will return back to the University of Dayton and work under Dr. Eric Benbow.

4. **Thornton, S., and J.K. Tomberlin. 2013.** Ms. Thornton was a junior microbiology major at Texas A&M University. During the summer of 2013, along-side PhD student, Chin Heo, she examined the microbial succession on swine carrion when arthropod activity was restricted. Stephanie will continue to work in the FLIES Facility, statistically analyzing the Biolog EcoPlate™ data collected to determine the relationship between microbial and arthropod activity on vertebrate carrion.

5. **Diaz, M. and M.E. Benbow. 2011.** Mr. Diaz was a sophomore majoring in Biology at the California State University in Monterey Bay. During the summer 2011 he spent 10 weeks in Dr. Benbow's lab doing research on how microbial metabolic profiles changed in response to food resources treated with antibiotics, examining how specific bacteria taxa are important to microbial function during succession and how blow fly larval growth and development respond to shifts in microbial metabolic profiles mediated by antibiotics.

6. **Henger, N., and M.E. Benbow. 2012-Present.** Ms. Henger is an undergraduate student at the University of Dayton. She has done research associated with sampling and analyzing microbial metabolic profiles from decomposing carrion. Additionally, she has worked directly with a postdoctoral associate, Dr. Jennifer Pechal, on collecting and identifying blow flies to better understand adult hyrocarbon profiles and internal microbiome.

7. **Caltaux, A., and M.E. Benbow. 2011-Present.** Ms. Caltaux is an undergraduate student at the University of Dayton. She has done research associated with sampling and analyzing microbial metabolic profiles from decomposing carrion and how scavengers affect the profiles.

8. **Shewhart, L., and M.E. Benbow. 2011.** Ms. Shewhart was an undergraduate student at the University of Dayton. She performed research associated with sampling and analyzing microbial metabolic profiles from decomposing carrion and how scavengers affected the profiles.

9. **Wright, A., and M.E. Benbow. 2011-Present.** Ms. Wright is an undergraduate student at the University of Dayton. She has done research associated with sampling and analyzing microbial metabolic profiles from decomposing carrion and how scavengers affect the profiles. She has also worked with a postdoctoral associate, Dr. Jennifer Pechal, on collecting and identifying blow flies to better understand adult hyrocarbon profiles and internal microbiome.

10. **Alfieri, J., and M.E. Benbow. 2012-2013.** Mr. Alfieri was an undergraduate student at the University of Dayton. He performed research associated with sampling and analyzing microbial metabolic profiles from decomposing carrion in relation to blow fly colonization. Additionally, he has worked directly with a postdoctoral associate, Dr. Jennifer Pechal, on collecting and identifying blow flies to better understand adult hyrocarbon profiles and internal microbiome.

11. **Whittaker, A., and M.E. Benbow. 2013-Present.** Ms. Whittaker is an undergraduate student at the University of Dayton. She has done research working with a postdoctoral associate, Dr. Jennifer Pechal, on collecting and identifying blow flies to better understand adult hyrocarbon profiles and internal microbiome from different ecoregions. She has also worked on understanding the effect of bait age on blow fly attraction.

12. **McHugh, M., and M.E. Benbow. 2013-Present.** Ms. McHugh is an undergraduate student at the University of Dayton. She has done research working with a postdoctoral associate, Dr. Jennifer Pechal, on collecting and identifying blow flies to better understand adult hyrocarbon profiles and internal microbiome from different ecoregions.

13. **Rhinesmith, J., and J.K. Tomberlin. 2012.** Ms. Rhinesmith was a senior majoring in Entomology at Texas A&M University. Jennie worked with Micah Flores in the FLIES Facility. She examined the role of quorum sensing molecules on the behavioral responses

of adult blow flies (Diptera: Calliphoridae) as well as the non-consumptive effects of *Chrysomya rufifacies* on *Cochliomyia macellaria*.

14. **Weldon, C., and J.K. Tomberlin. 2012.** Ms. Weldon was an undergraduate Forensic & Investigative Sciences student in the Department of Entomology at Texas A&M University., Courtney isolated and described the bacteria fauna associated with the salivary glands of the blow fly *Lucilia sericata* (Diptera: Calliphoridae). She also examined the role of quorum sensing by bacteria as a mechanism regulating adult blow fly behavior.

15. **Adams, K., and J.K. Tomberlin. 2011.** Ms. Adams was a senior in the Forensic and Investigative Sciences major at Texas A&M University. Her research examined trophic interactions between blow flies and microbes associated with carrion. Specifically, she examined the attraction and oviposition responses of adult *L. sericata* to strains of *P. mirabilis* which is a microbe commonly associated with decomposition.

16. **Diaz, M. and J.K. Tomberlin. 2010.** Mr. Diaz was a sophomore majoring in Biology at the California State University in Monterey Bay. During the summer 2010 Michael investigated the relationship between blow fly (Diptera: Calliphoridae) larval size and time of dispersal from carrion. Michael determined that those initially dispersing from carrion are larger than those dispersing at a later time indicating that those colonizing late are penalized in their ability to gain weight (i.e., greater larval competition for limited resources). Consequently, less weight could translate into reduced ability to survive, mate, and reproduce. This information has forensic significance as larval size (i.e., length and weight) are often used to estimate time of colonization. Failure to recognize the phenotypic variation existing within a species could result in an over, or under, estimate of the time of colonization.

Accomplishment #4. Graduate student training.

1. **Sonja Stadler. 2013.** Ph.D Dissertation: Analysis of the volatile organic compounds produced by the decomposition of pig carcasses and human remains. Department of Applied Sciences, University of Ontario Institute of Technology.

2. **Micah Flores. 2013.** Ph.D. Dissertation: Life-history traits of *Chrysomya rufifacies* (Macquart) (Diptera: Calliphoridae) and its associated non-consumptive effects on *Cochliomyia macellaria* (Fabricius) (Diptera: Calliphoridae) behavior and development. Department of Entomology, Texas A&M University, College Station, Texas.

3. **Jennifer Pechal**. **2012.** Ph.D. Dissertation: Swine carrion as a model system for studying decomposition ecology: The importance of microbial and primary colonizer interactions. Department of Entomology, Texas A&M University, College Station, Texas.

4. **Adrienne Brundage**. **2012.** Ph.D. Dissertation: Bacterial mediated intra- and interspecific interactions in the Calliphoridae with special reference to forensically important species. Department of Entomology, Texas A&M University, College Station, Texas.

Accomplishment #5: Postdoctoral training.

1. **Postdoctoral associate, Dr. Jennifer Pechal**, has been a scientific mentor and supervisor of undergraduate research assistants associated with accomplishing Objective #1. She also has mentored a middle school student on two science fair projects and given guest lectures at the University of Dayton. There have been eight undergraduate students that have been trained at the University of Dayton. These students participated in research that supported Objective #1.

2. **Postdoctoral Associate: Dr. Baneswhar Singh** attended the National Science Foundation (Grant # DEB 0733029) sponsored workshop entitled, "Symposium and Workshop on New Methods for Phylogenomics and Metagenomics." University of Texas, Austin, Texas. This work was in support of Objective #1.

Accomplishment #6: Awards.

1. **Sandler, S. 2012.** First place award for the following presentation by S. Stadler: Did Halloween scare away the VOCs? An investigation into the production of VOCs from human decomposition. 3rd Annual UOIT Graduate Student Research Conference. Oshawa, Ontario, Canada.

2. **Rhinesmith, J. 2012.** Quorum sensing by *Escherichia coli* serves as interkingdom signal with *Lucilia sericata* (Diptera: Calliphoridae). 1st place, non-PhD student.10th Annual North American Forensic Entomology Conference, Las Vegas, Nevada.

3. **Diaz, M. 2011.** Pupal size throughout dispersal of the secondary screwworm, *Cochliomyia macellaria* (Diptera: Calliphoridae): implications for forensic entomology. 1st place, non-PhD student. 9th Annual North American Forensic Entomology Conference, College Station, Texas.

Accomplishment #7: Additional funding received ($139,627.75).

1. **Tarone, A.M., and J.K. Tomberlin. 2013.** Whole Systems Genomics Initiative Traineeship. **$11,002.75.** Assistance for Ms. Meaghan Pimsler. Texas A&M University.

2. **Tomberlin, J.K. and A.M. Tarone. 2013.** Texas Invasive Ant Research and Management Seed Grant Program. **$118,500.00.** Deciphering interkingdom communication between bacteria and red imported fire ants to develop novel bait attractants and ant repellants. Research generated from our NIJ grant allowed us to continue to investigate the role of microbes as a mechanism regulating arthropod attraction to food resources. Fire ants are commonly associated with human remains. Thus, the research we are now conducting as a result of our NIJ grant will over greater insight to how microbes regulate arthropod colonization of human remains.

3. **Tomberlin, J.K., and A.M. Tarone. 2010.** Whole Systems Genomics Initiative Traineeship. **$7,125.00.** Texas A&M University. Assistance for Ms. Jennifer Pechal. Texas A&M University. These funds provided salary for Ms. Pechal as she completed her PhD examining microbial community structure and function associated with

vertebrate carrion. Her research served as the foundation on which our grant was developed. Through her research we were able to develop the methods that were employed with our NIJ grant.

4. **Benbow, M.E., J.R. Wallace, J.L. Pechal, J.M. Lang. 2012.** American Academy of Forensic Sciences (Pathology/Biology Section Grant). **$3,000.00.** Aquatic microbial communities to estimate the postmortem submersion interval. Research generated from our NIJ grant allowed us to demonstrate that pyrosequencing could be used for assessing postmortem submersion intervals of corpses and carrion found in aquatic habitats.

Accomplishment #8: Collaboration developed due to grant.

1. **Mike Allen, Assistant Professor, Department of Biological Sciences, University of North Texas.** Dr. Allen provided sequencing of *Proteus* and *Providencia* strains isolated from *L. sericata*.

2. **Helene LeBlanc, Assistant Professor, Faculty of Science, University of Ontario, Institute of Technology.** In order to develop a link between microbial activity on human remains and arthropod attraction, Dr. LeBlanc attempted to capture and quantify volatile organic compounds produced. She used two techniques to capture these compounds and analyzed the collected compounds using GC-MS. She is now analyzing these data to determine their role in arthropod attraction. She will continue her research using electroantennagram technology to determine which odors stimulate a neural response in a model blow fly species associated with carrion.

3. **Shari Forbes Assistant Professor, Faculty of Science, University of Ontario, Institute of Technology.** Dr. Forbes worked with Sonja Stadler to also quantify volatile organic compound production associated with humans. Her efforts were similar to those of Dr. LeBlanc; however, she was using a different technique to assess these volatiles.

4. **Jacqui Aitkenhead-Peterson, Assistant Professor, Department of Soil & Crop Sciences, Texas A&M University.** Soil and geologic characteristics associated with soil can be used to determine the length of time the remains have been present at a given site. Dr. Peterson examined the soil chemistry to determine its relationship with the microbial community structure and function.

5. **Mike Strickland, Assistant Professor, Department of Biology, Virginia Tech University.** Much like Dr. Peterson, Dr. Strickland was also examining the soil chemistry associated with the human remains studied as part of our grant. He examined the nitrogen: carbon ratio to determine its relationship with the amount of time to elapse since the placement of the remains in the field.

6. **Danny Wescott, Associate Professor, Department of Anthropology, Texas State University.** Dr. Wescott is the Director for the Forensic Anthropology Research Facility at Texas State University. He allowed us to conduct our research at his facility.

7. **Rodney Rohde, Professor & Chair, Clinical Laboratory Science Program, Texas State University.** We attempted to develop cross-training with students at Texas State

University. Through these interactions, three undergraduate students associated with this program collaborated on our research efforts. We also were allowed to use laboratory space provided by Dr. Rohde

8. **Kyle Wickings, Postdoctoral Associate, Department of Natural Resources, University of New Hampshire.** Dr. Wickings examined the soil micro-arthropod fauna associated with the human remains studied as part of our grant. His research objective was to determine the relationship between soil mirco-arthropods, chemistry, nutrients, and microbial communities in order to estimate the time since placement of the remains in the field.

9. **Hannah E. Moore and Falko P. Drijfhout, Keele University, United Kingdom.** Drs. Moore and Drijfhout have worked with Drs. Benbow and Pechal on understanding the cuticular hydrocarbon profiles of field captured adult blow flies.

Accomplishment #9: Opportunities for training and professional development.

1. **Tomberlin, J.K. J. Mayers, and B. Singh. 2013.** Integration of Veterinary Medicine and Entomology in Forensics. 2013. School of Veterinary Medicine, Texas A&M University.

2. **A.M. Tarone.** The genomics of blow fly development: Advancing research in forensic science and sex determination. Texas Genetics Society. College Station, TX, May 2013.

3. **A.M. Tarone.** Proper methods for collecting insects from a crime scene. TEEX Skeletal Death Investigation Course. Forensic Anthropology Center, Texas State University, San Marcos, TX. May 21, 2013.

4. **A.M. Tarone, A. Faris, and J. Parrott.** Forensic Entomology in Forensic Taphonomy. Forensic Taphonomy Workshop. Forensic Anthropology Center, Texas State University, San Marcos, TX. June 11, 2013.

5. **Tomberlin, J.K. 2012.** New trends in forensic entomology. 75th Texas International Association for Identification. Galveston, Texas.

6. **Tomberlin, J.K. Pechal, J.L., and B. Singh. 2012.** Skeletal Death Investigation. Texas Engineering Extension Service. 2012. Texas State University.

7. **Tomberlin, J.K., M. Flores, and J. Pechal. 2012.** Forensic entomology workshop. San Antonio Police, San Antonio, Texas.

8. **Tomberlin, J.K. 2012.** Forensic Entomology. San Antonio Police Department, San Antonio, Texas.
9. **Tomberlin, J.K., B. Singh, M.L. Pimsler, C. Owings, and M. Flores. 2012.** Forensic Entomology. 2012. Evidence Response Team, Federal Bureau of Investigation, Houston, Texas.

10. **Pechal, JL, ME Benbow. 2012.** *An Introductory Manual for Multivariate Statistical Analysis of Community Data Using PCORD*. Manual for North American Forensic Entomology Association Workshop. Las Vegas, NV, 17 July 2012.

11. **Tomberlin, J.K. 2010.** Evidence Recovery Team: Federal Bureau of Investigation. Department of Anthropology, University of Tennessee, Knoxville, Tennessee.

Accomplishment #10: Outreach activities to the public.

1. **Benbow, M.E. and J.L. Pechal**. 2013. ShamROCK Science Day, Boonshoft Museum of Discovery.

2. **Benbow, M.E. and J.L. Pechal**. 2013. Family Science Fest, Liebold Catholic High School.

3. **Benbow, M.E. and J.L. Pechal**. 2012. STEMM Celebration for Families, Ignite Innovation, Dayton Regional Science Festival, Chaminade Julienne Catholic High School.

4. **Benbow, M.E. and J.L. Pechal. 2012** Chemistry and Cocktails, Ignite Innovation, Dayton Regional Science Festival, Boonshoft Museum of Discovery.

TABLES AND FIGURES

Table 1. Odors released by microbes and known to attract blow flies (Diptera: Calliphoridae)*
Indole
Skatole
Paracresol
Phenol
Volatile fatty acid
Benzoic acid
Indol-acetic acid
Methyl mercaptan
Hydrogen sulfide
Methane

*Dethier, V.G. 1947. Chemical Attractants and Repellents. The Blackiston Company, Philadelphia, PA. 289 pp.

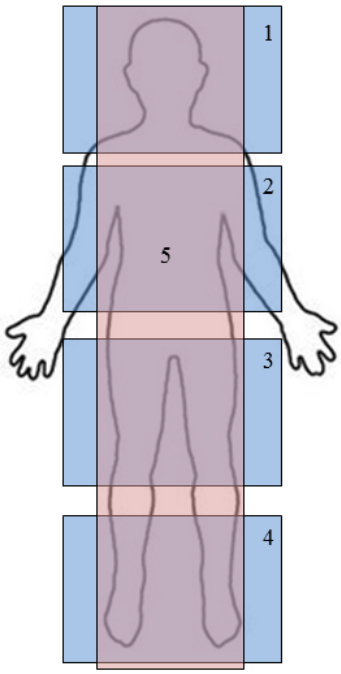


Figure 1. Diagram of proposed sites for microbial sampling from human remains.

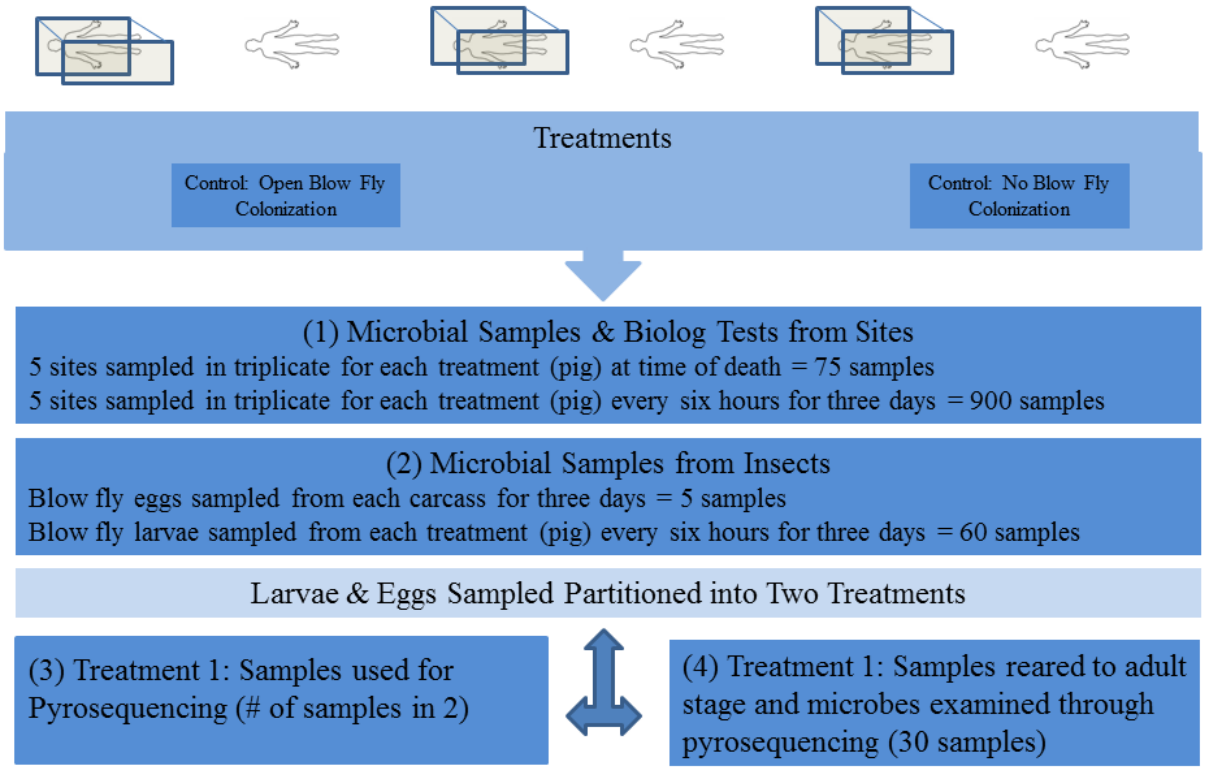


Figure 2. Diagram of proposed microbial sampling developed for this research project.

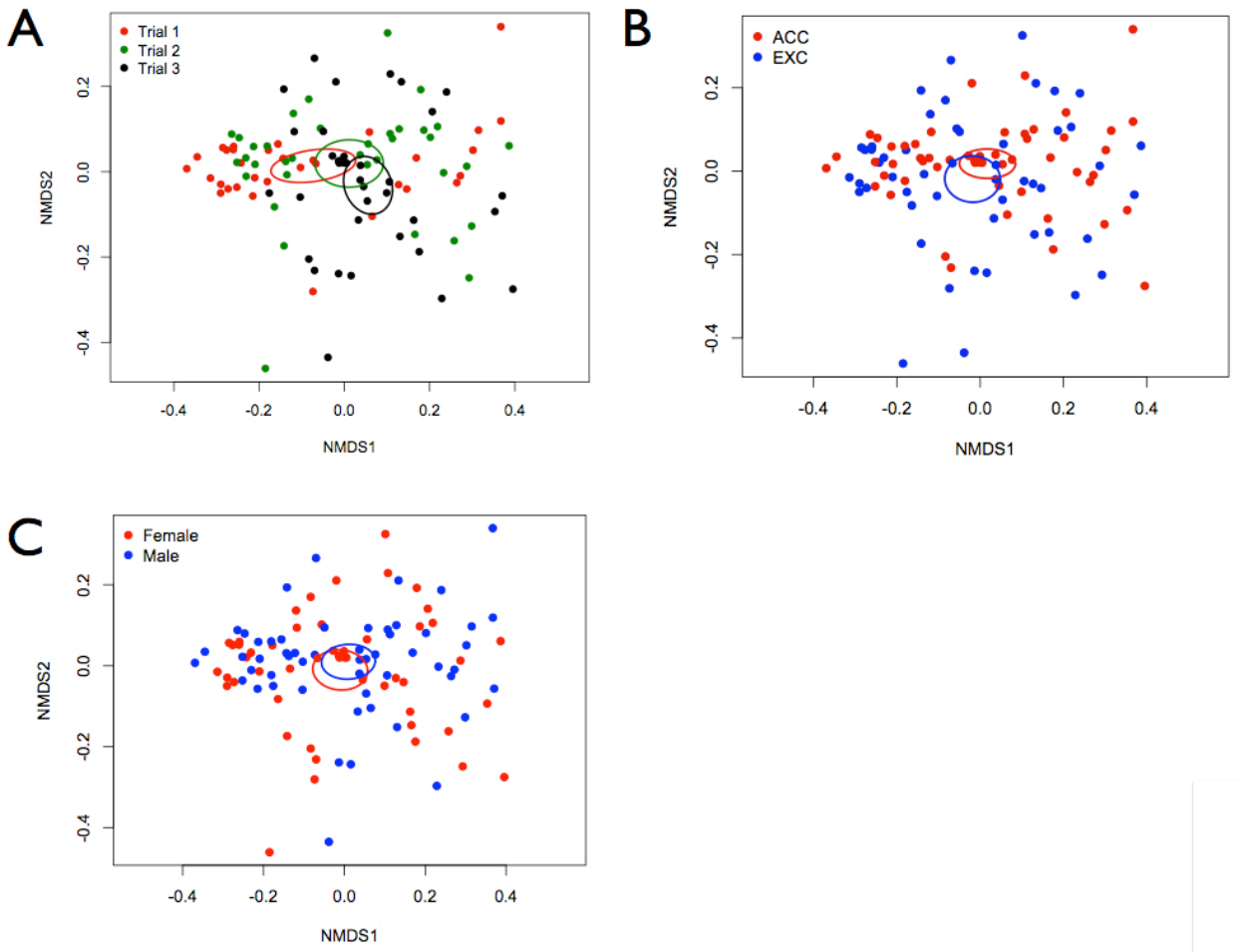


Figure 3. Biolog- Non-metric multidimensional scaling ordinations of MMCPs were plotted on two dimensions (stress = 0.189, R^2 = 0.841) for human communities among field trials 1-3 to assess A) trial, B) treatment and C) sex differences. There were significant differences among trials (PERMANOVA: $P < 0.001$); no significant differences between treatment or sex, and a significant interaction (P=0.008); no significant difference between treatment or among sampling days, and no significant interaction; and no significant difference between sex or among sampling days, and no significant interaction. The circles indicate 95% standard error of the mean.

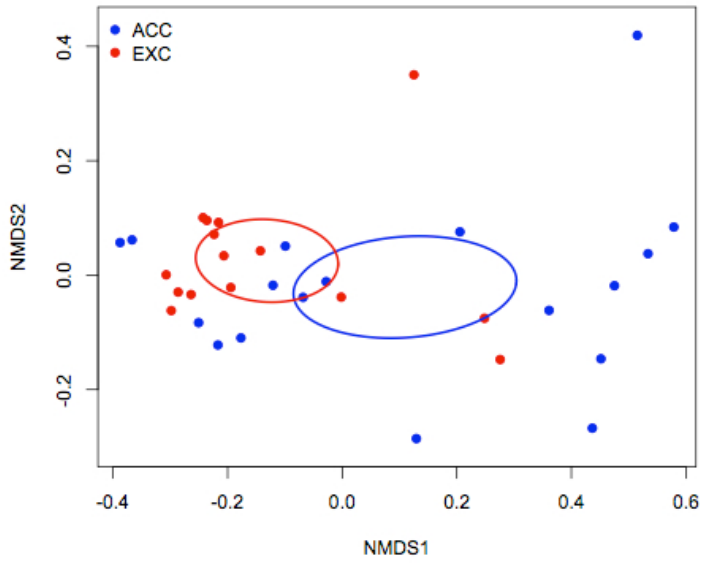


Figure 4. Biolog- Non-metric multidimensional scaling ordinations of MMCPs were plotted on two dimensions (stress = 0.094, R^2= 0.970) for human communities (H1 and H2) in trial 1. There were significant differences between treatments (PERMANOVA: P = 0.015). The circles indicate 95% standard error of each treatment mean.

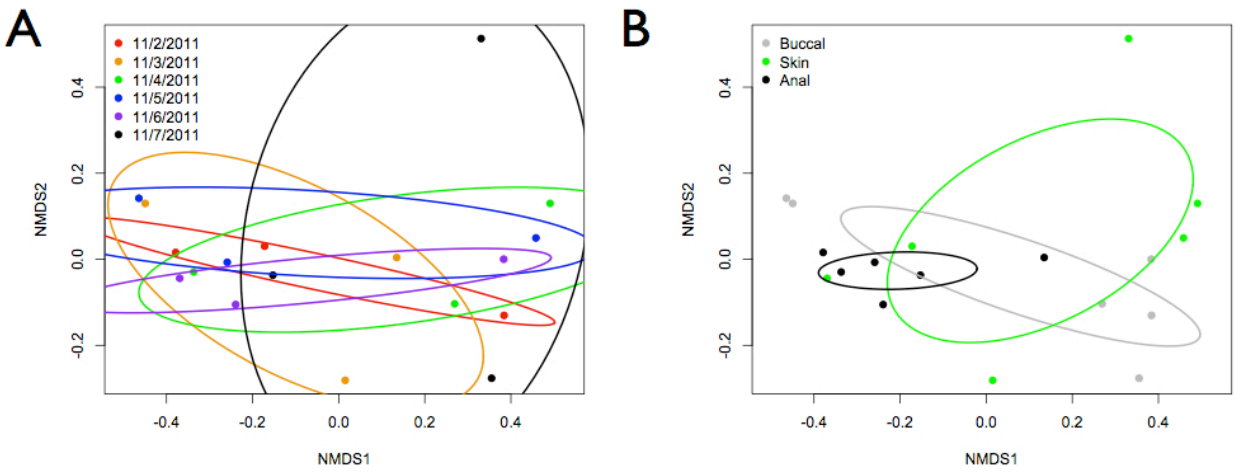


Figure 5. Biolog- Non-metric multidimensional scaling ordinations of MMCPs were plotted on two dimensions (stress = 0.077, R^2 = 0.975) for ACC human communities (H1) in trial 1 to assess A) sampling day and B) sampling area differences. There were no significant differences among sampling days, sampling area (buccal, skin, anal) and no significant interaction. The circles indicate 95% standard error of the mean.

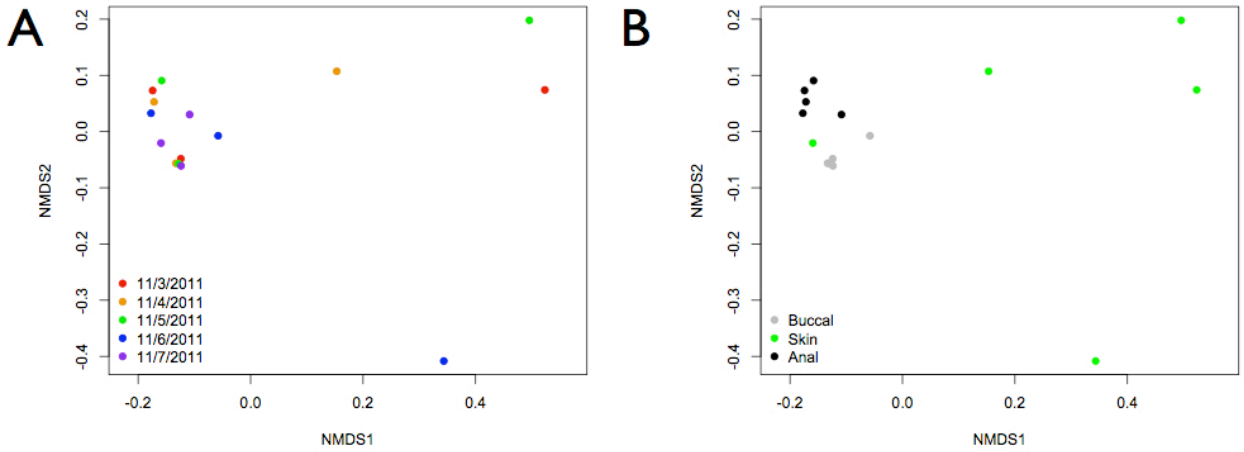


Figure 6. Biolog- Non-metric multidimensional scaling ordinations of MMCPs were plotted on two dimensions (stress = 0.046, $R^2 = 0.995$) for EXC human communities (H2) in trial 1. There were no significant differences among sampling days, sampling area and no significant interaction. However, there was a significant difference among sampling sties without account for day of decomposition (PERMANOVA: $P < 0.001$). The circles indicate 95% standard error of the mean.

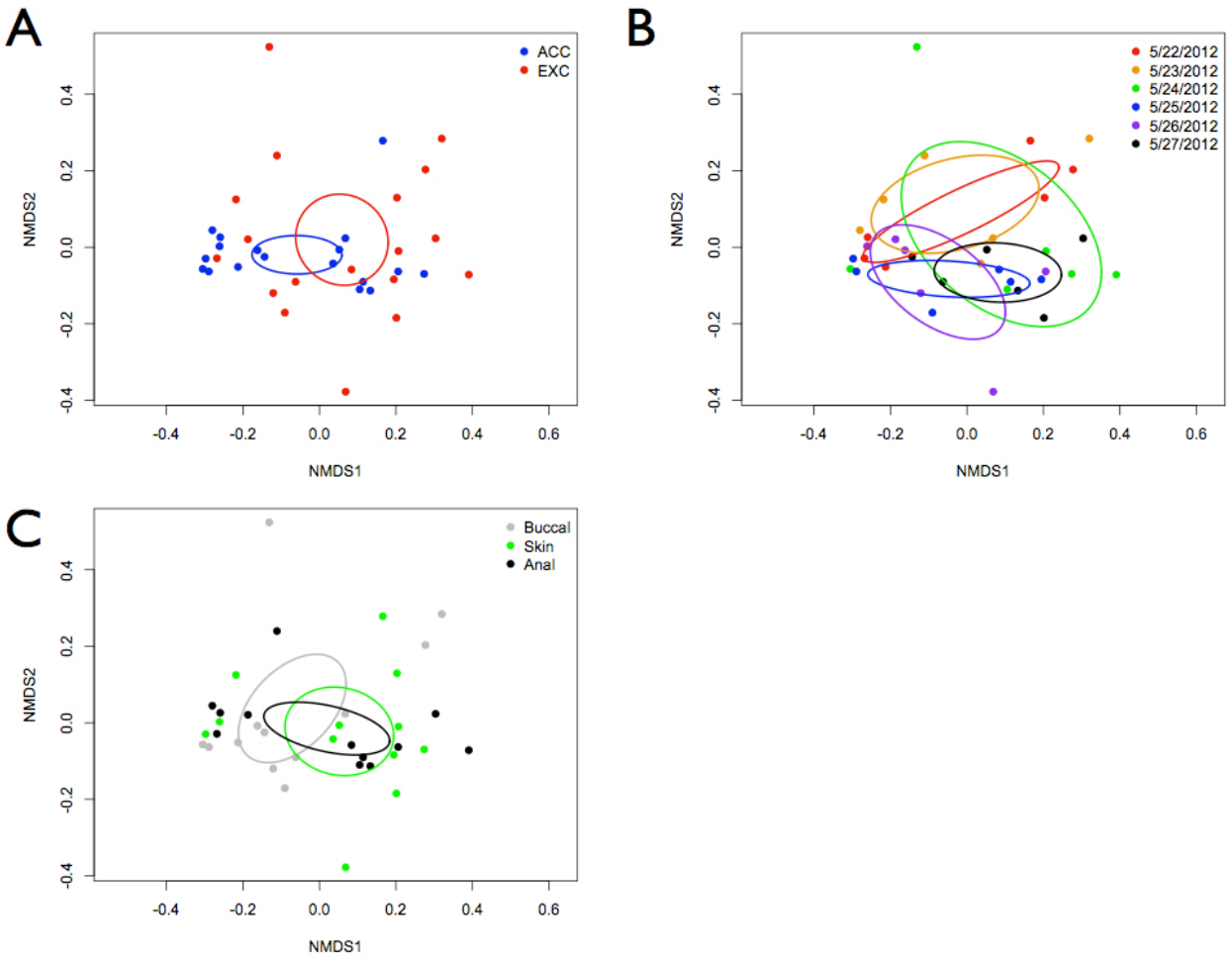


Figure 7. Biolog- Non-metric multidimensional scaling ordinations of MMCPs were plotted on two dimensions (stress = 0.148, R^2= 0.902) for human communities (H3 and H4) in trial 2. There were no significant differences between treatments, sampling day, and no significant interaction. Furthermore, there were no significant differences between sampling area, sampling day, and no significant interaction. The circles indicate 95% standard error of the mean.

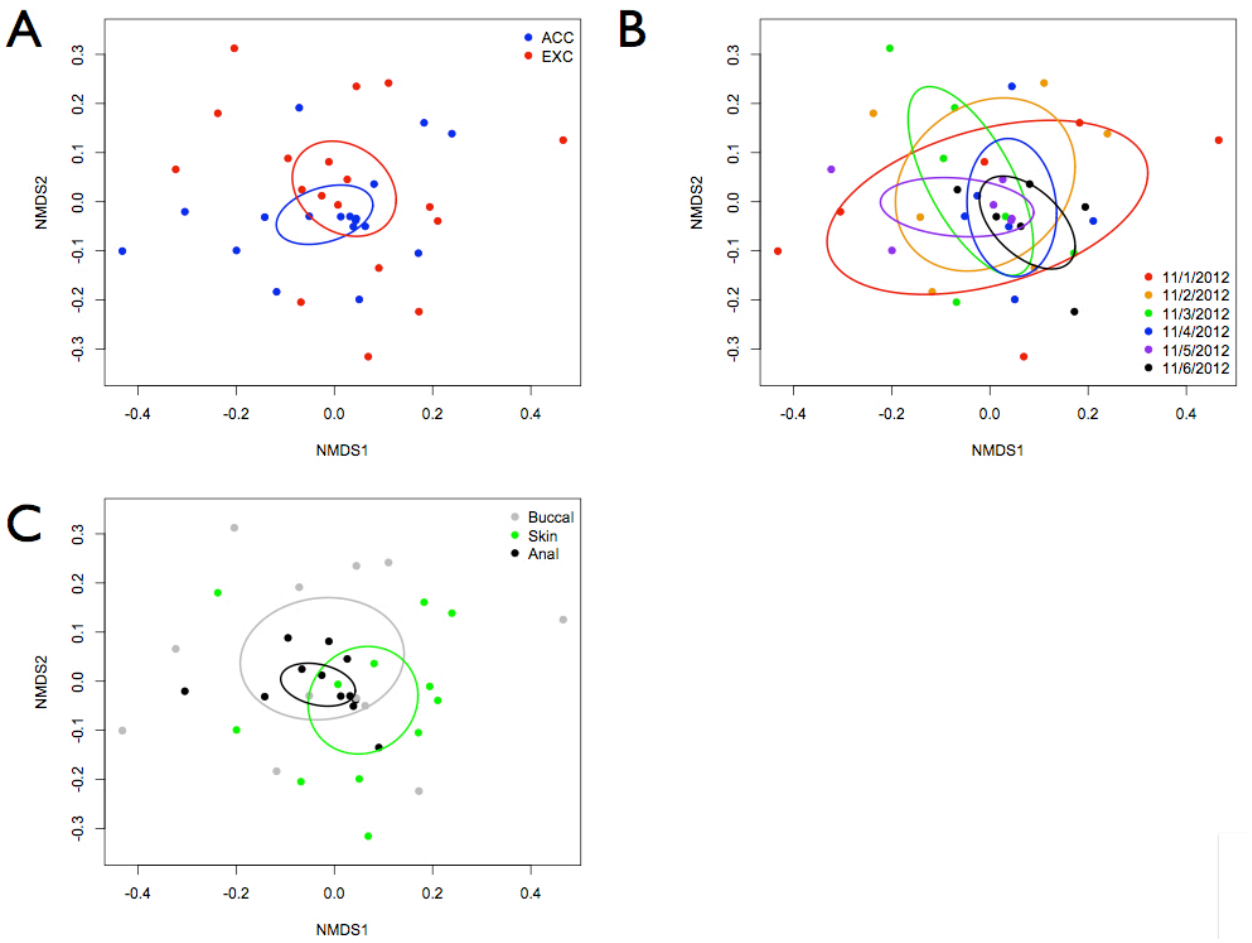


Figure 8. Biolog- Non-metric multidimensional scaling ordinations of MMCPs were plotted on two dimensions (stress = 0.216, R^2 = 0.795) for human communities (H5 and H6) in trial 3. There were no significant differences among A) treatments; no significant differences between B) no significant difference between treatment or among sampling days, and no significant interaction; and no significant difference among C) sampling areas or among sampling days, and no significant interaction. The circles indicate 95% standard error of the mean.

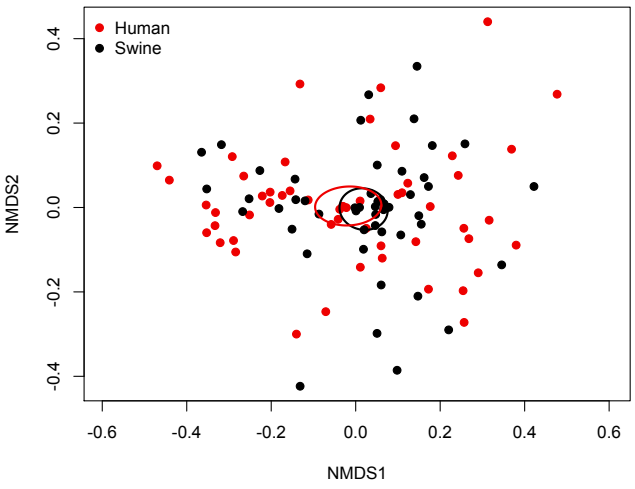


Figure 9. Biolog- Non-metric multidimensional scaling ordinations of MMCPs were plotted on two dimensions (stress = 0.189 R^2= 0.856) for ACC human communities (H1, H3, and H5) and swine communities (P1-P3) in all field trials. There were significant differences between treatments (PERMANOVA: P = 0.006). The circles indicate 95% standard error of each species mean.

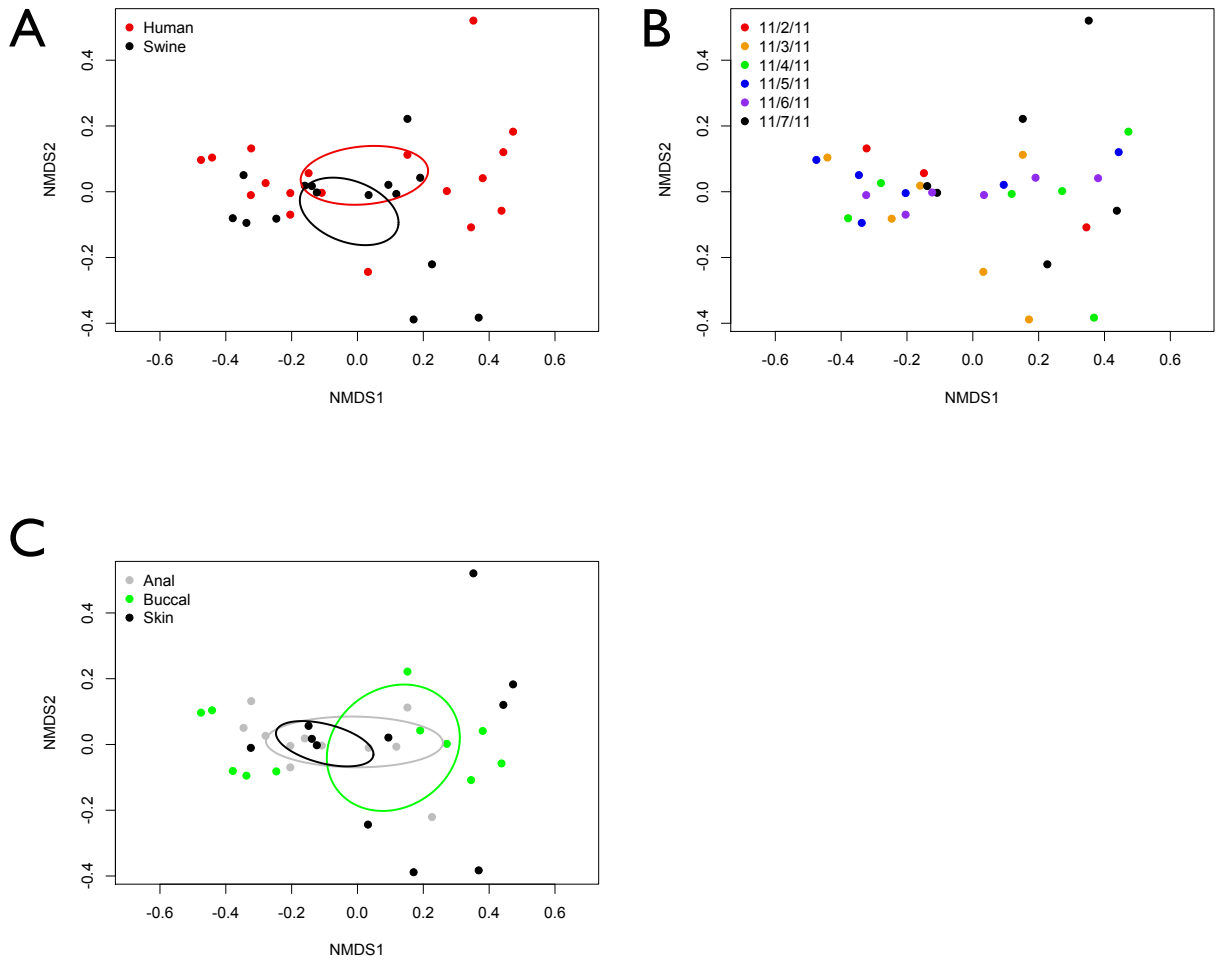


Figure 10. Biolog- Non-metric multidimensional scaling ordinations of MMCPs were plotted on two dimensions (stress = 0.113, R^2 = 0.947) for ACC human communities (H1) and swine communities (P1) in field trial 1. There were no significant differences among A) species; no significant differences between B) no significant difference between species or among sampling days, and no significant interaction; and no significant difference among C) sampling areas or among sampling days, and a significant interaction (P = 0.030). The circles indicate 95% standard error of the mean.

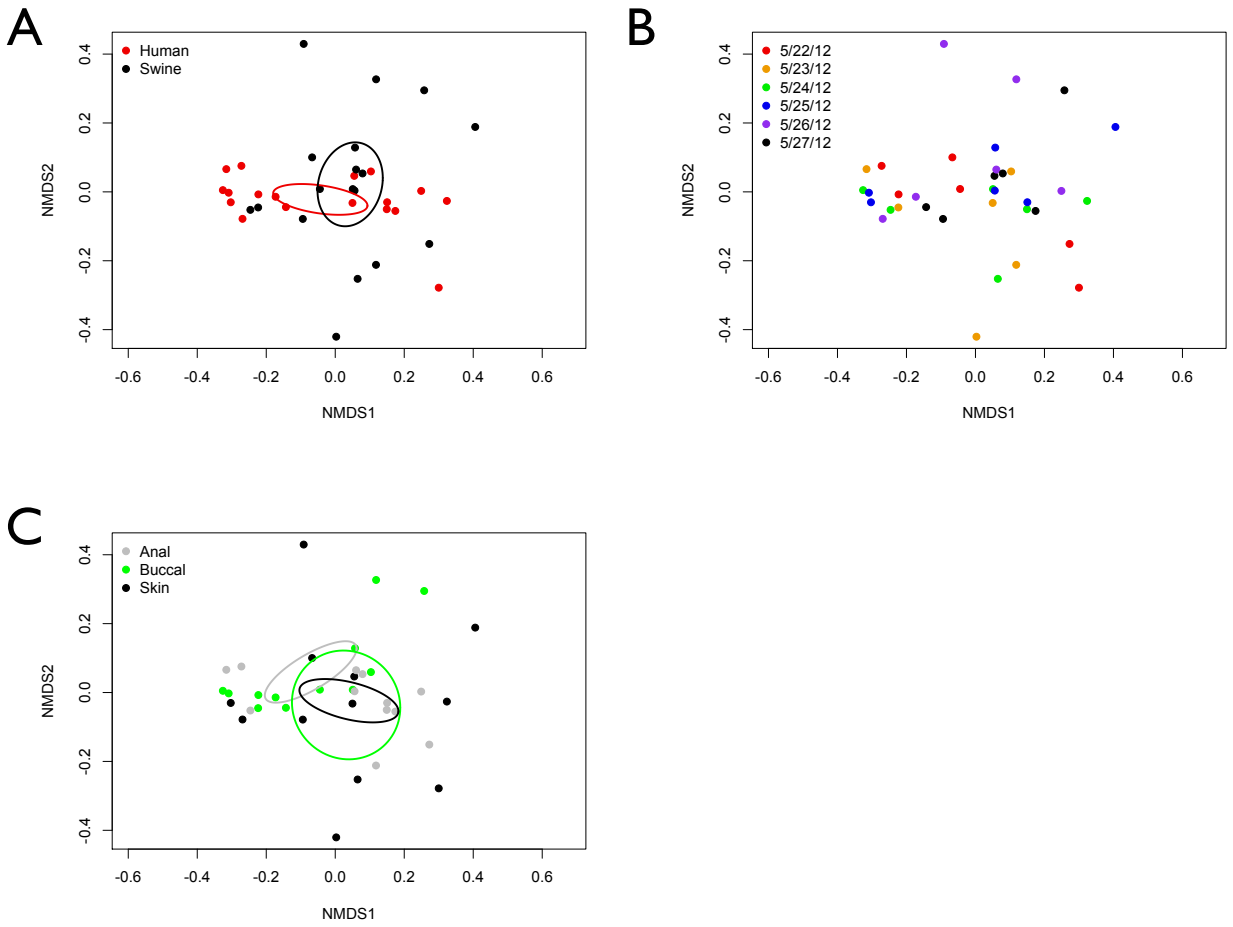


Figure 11. Biolog- Non-metric multidimensional scaling ordinations of MMCPs were plotted on two dimensions (stress = 0.159, R^2 = 0.886) for ACC human communities (H3) and swine communities (P2) in field trial 2. There were no significant differences among A) species; no significant differences between B) no significant difference between species or among sampling days, and no significant interaction; and no significant difference among C) sampling areas or among sampling days, and no significant interaction. The circles indicate 95% standard error of the mean.

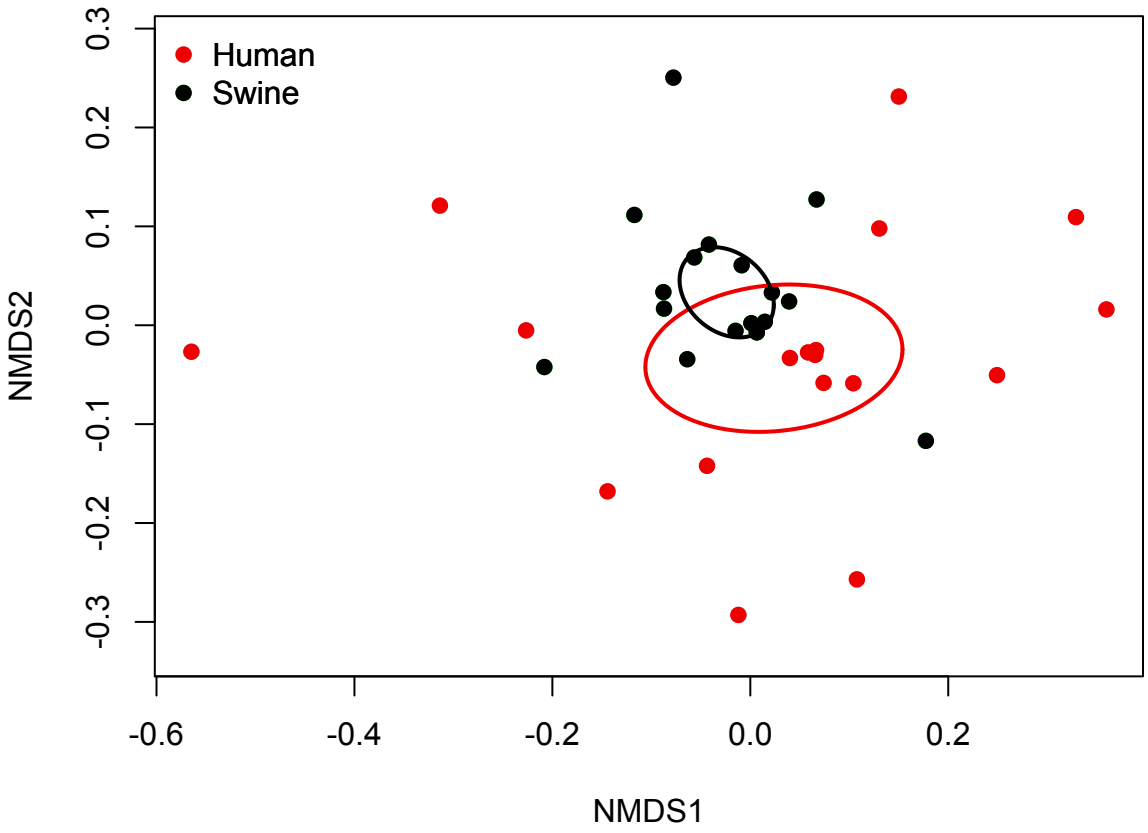


Figure 12. Biolog- Non-metric multidimensional scaling ordinations of MMCPs were plotted on two dimensions (stress = 0.170, R^2 = 0.910) for ACC human communities (H5) and swine communities (P3) in field trial 3. There were significant differences among species (P = 0.004) The circles indicate 95% standard error of the mean.

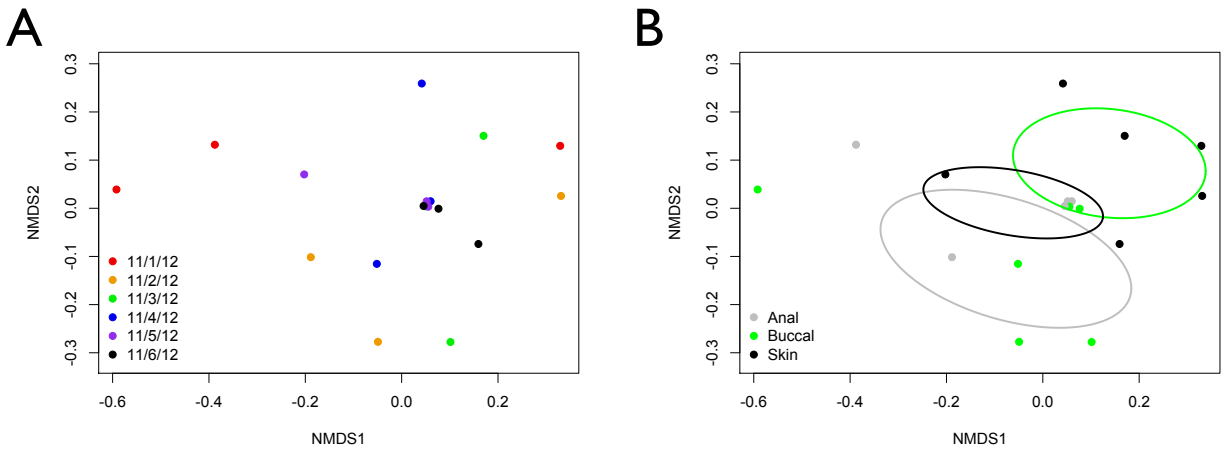


Figure 13. Biolog- Non-metric multidimensional scaling ordinations of MMCPs were plotted on two dimensions (stress = 0.121, R^2 = 0.945) for ACC human communities (H5) in field trial 3. There were no significant differences among A) sampling areas or B) among sampling days, and no significant interaction. The circles indicate 95% standard error of the mean.

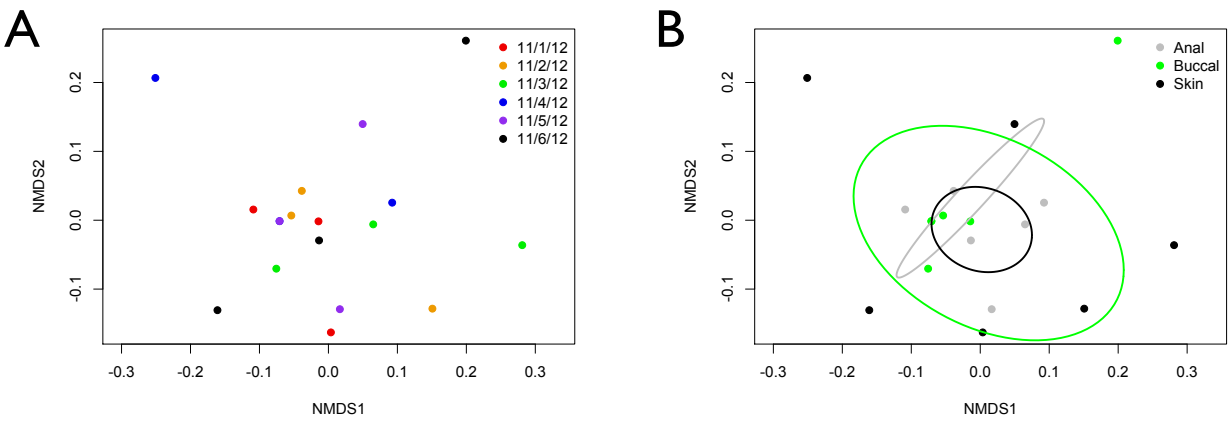


Figure 14. Biolog- Non-metric multidimensional scaling ordinations of MMCPs were plotted on two dimensions (stress = 0.134, R^2 = 0.929) for swine communities (P3) in field trial 3. There were no significant differences among A) sampling areas or B) among sampling days, and no significant interaction. The circles indicate 95% standard error of the mean.

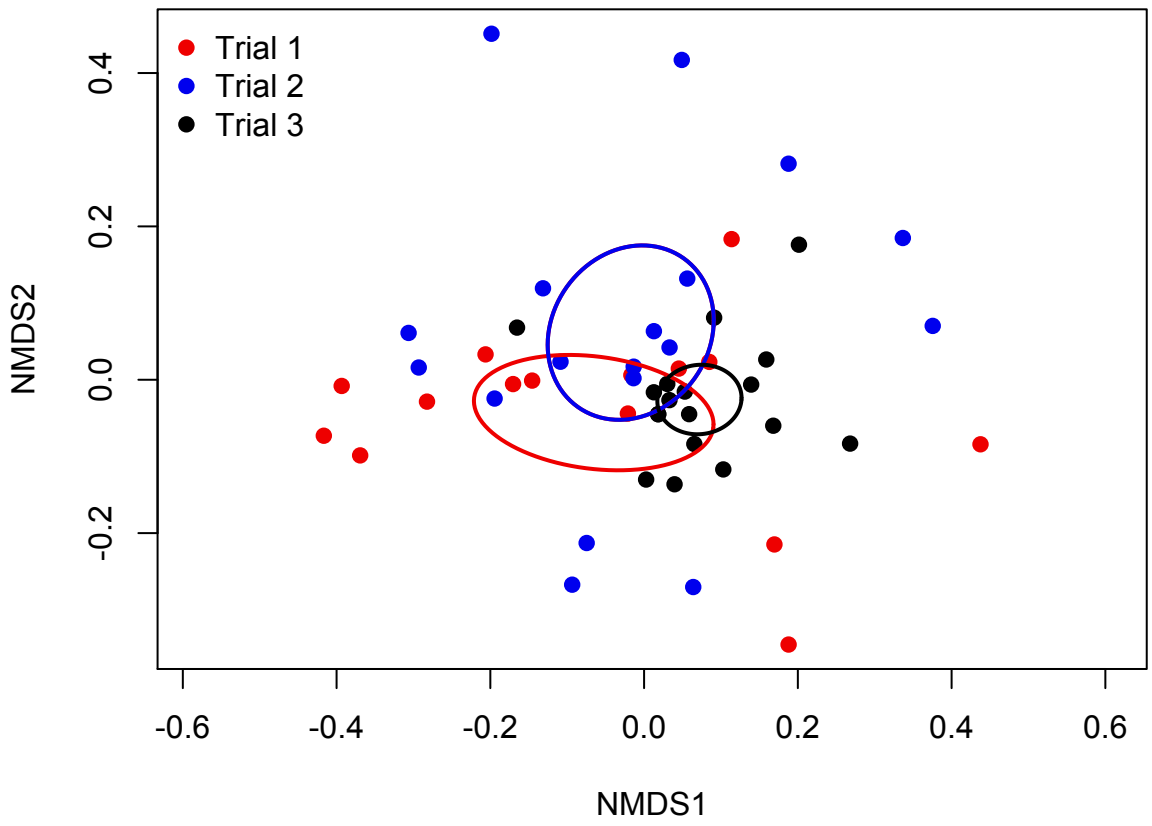


Figure 15. Biolog- Non-metric multidimensional scaling ordinations of MMCPs were plotted on two dimensions (stress = 0.169, R^2 = 0.885) for swine communities (P1-P3) in all field trials. There were significant differences among trials ($P < 0.001$) The circles indicate 95% standard error of the mean.

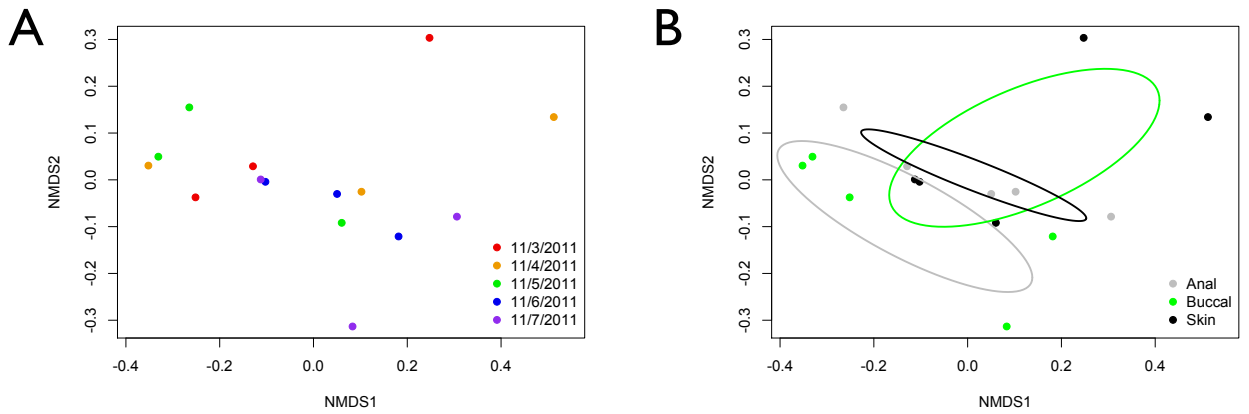


Figure 16. Biolog- Non-metric multidimensional scaling ordinations of MMCPs were plotted on two dimensions (stress = 0.078, R^2 = 0.973) for swine communities (P1) in field trial 1. There were no significant differences among A) sampling areas or B) among sampling days, and no significant interaction. The circles indicate 95% standard error of the mean.

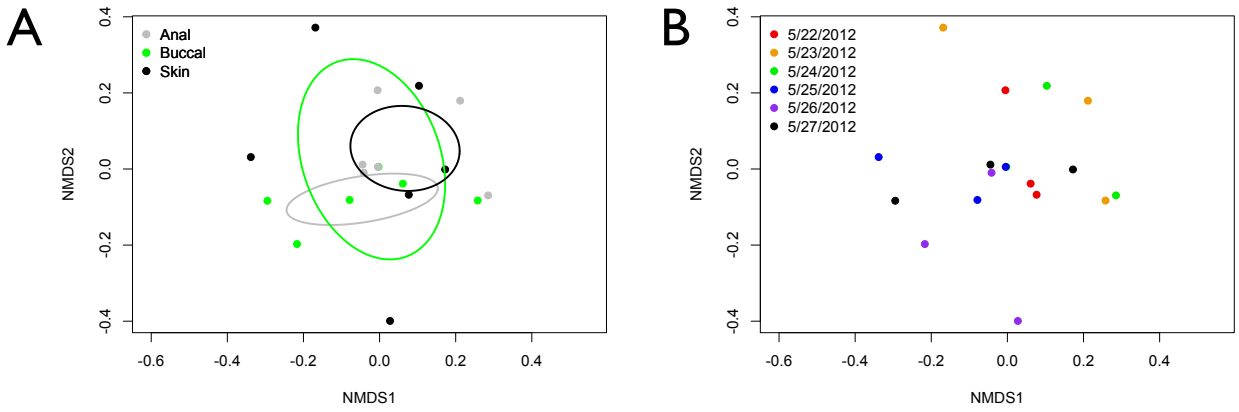


Figure 17. Biolog- Non-metric multidimensional scaling ordinations of MMCPs were plotted on two dimensions (stress = 0.154, R^2 = 0.883) for swine communities (P2) in field trial 2. There were no significant differences among A) sampling areas or B) among sampling days, and no significant interaction. The circles indicate 95% standard error of the mean.

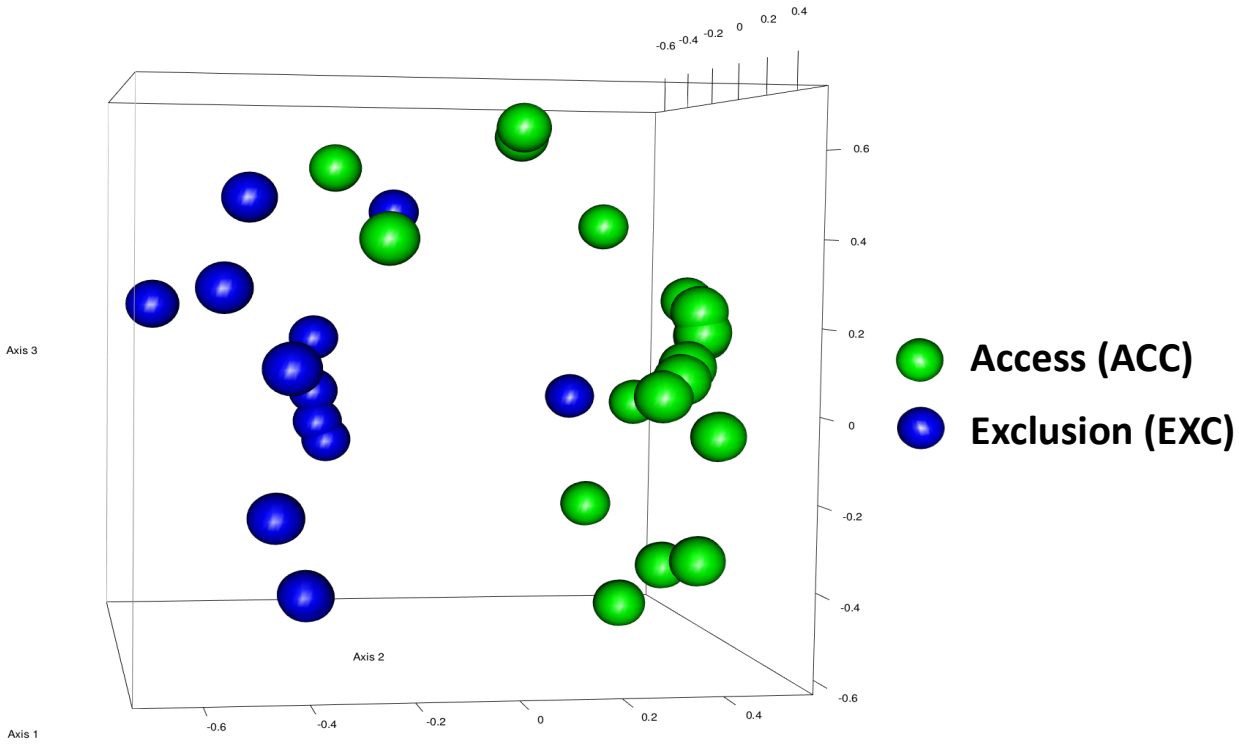


Figure 18. Three dimensional non-metric multidimensional scaling (NMDS) of pyrosequencing data from human cadavers (H1 and H2) in trial 1. There were significant differences between treatments (Insect access versus insect exclusion: AMOVA $P = < 0.0002$).

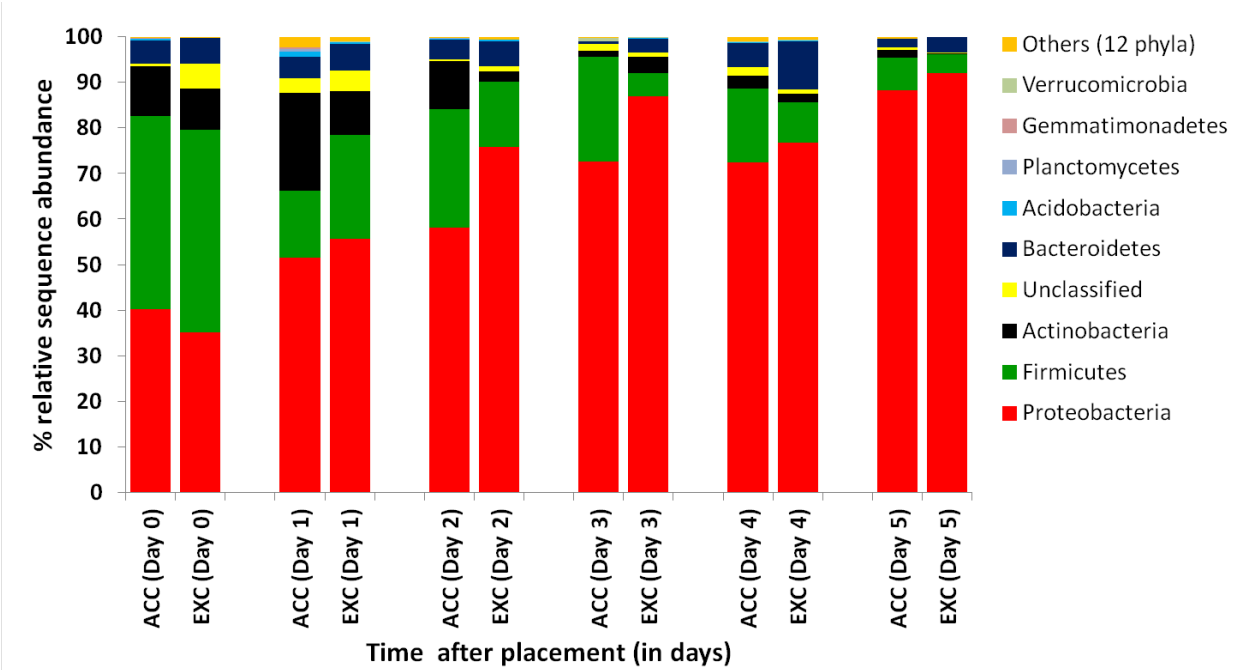


Figure 19. Bar diagram of temporal phylum level bacterial diversity in insect access (ACC) and insect exclusion (EXC) human cadavers based on pyrosequencing data.

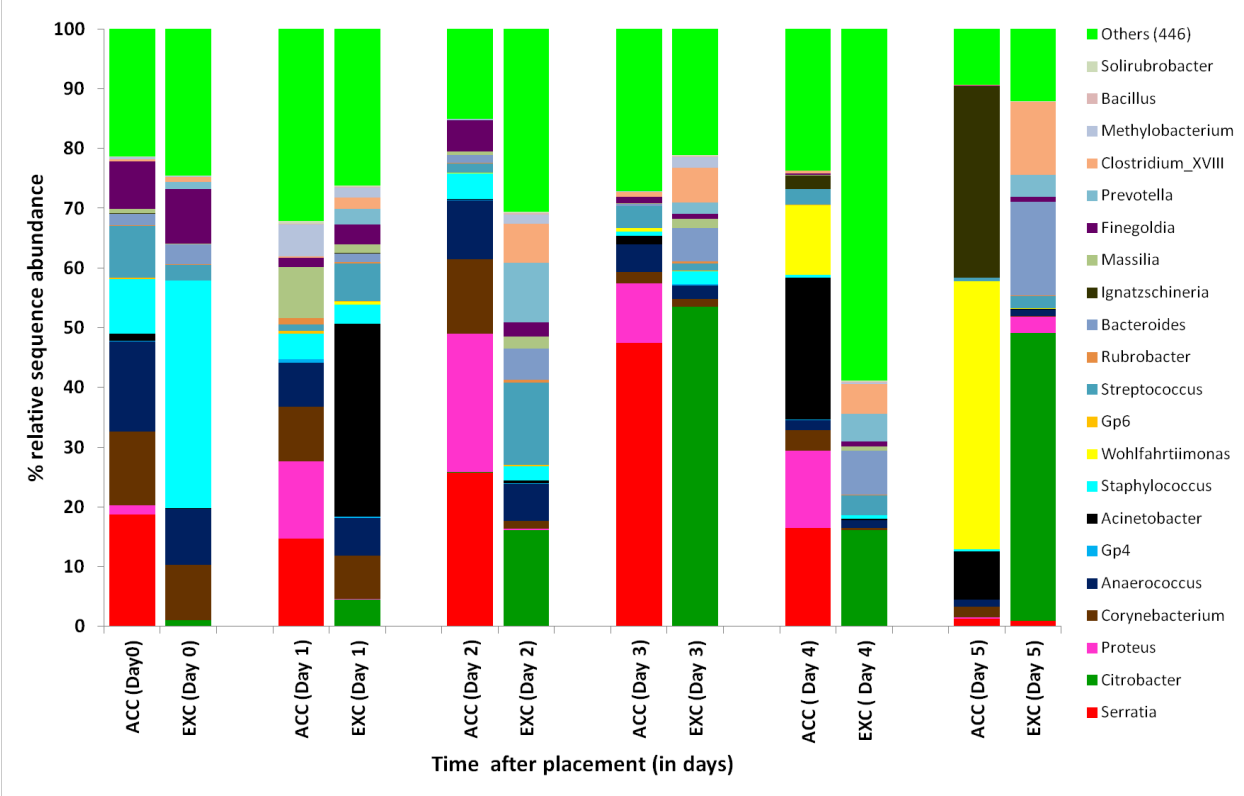
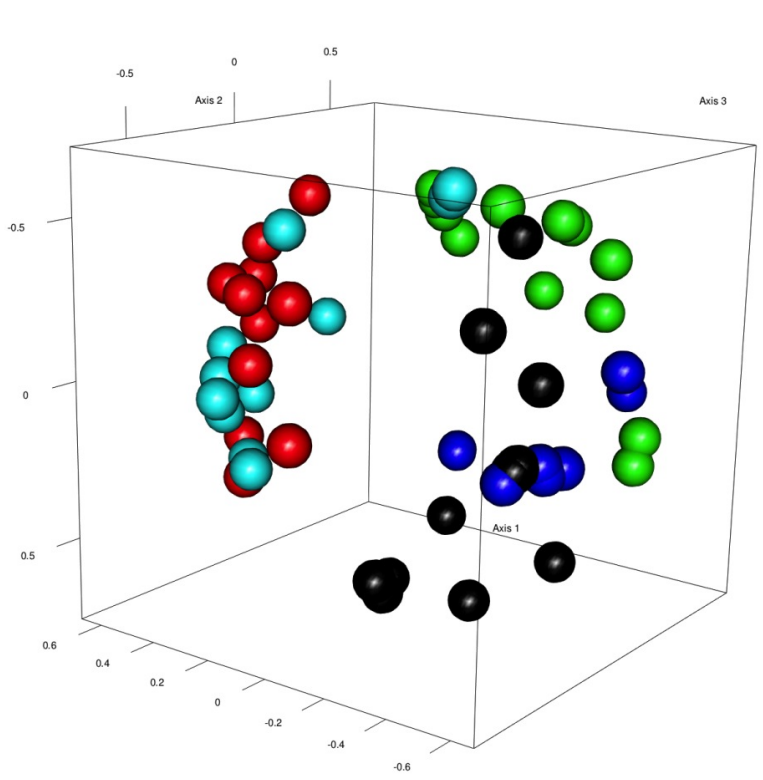


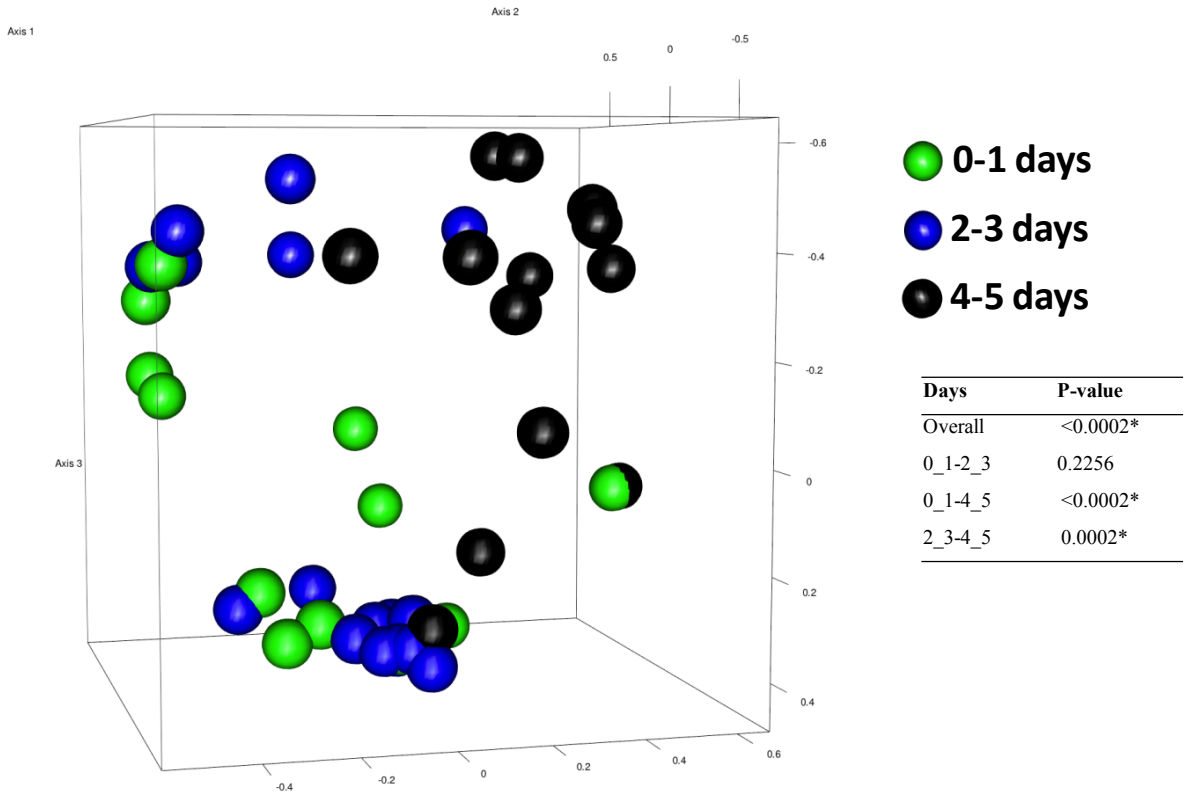
Figure 20. Bar diagram of temporal genus level bacterial diversity in insect access (ACC) and insect exclusion (EXC) human cadavers based on pyrosequencing data.



Anal
Buccal
Skin
Soil 1m away
Soil under the body

Treatments	p-value
All	<0.0002*
Anal-Buccal	<0.0002*
Anal-Skin	<0.0002*
Anal-Soil_1m_away	<0.0002*
Anal-Soil_under	<0.0002*
Buccal-Skin	0.0428
Buccal-Soil_1m_away	<0.0002*
Buccal-Soil_under	<0.0002*
Skin-Soil_1m_away	<0.0002*
Skin-Soil_under	0.0002*
Soil_1m_away-Soil_under	0.0748

Figure 21. Three dimensional non-metric multidimensional scaling (NMDS) of pyrosequencing data from human cadavers (H1 and H2) in trial 1. There were significant differences between body sites and also between body sites and soil samples.



Days	P-value
Overall	<0.0002*
0_1-2_3	0.2256
0_1-4_5	<0.0002*
2_3-4_5	0.0002*

Figure 22. Three dimensional non-metric multidimensional scaling (NMDS) of pyrosequencing data from human cadavers (H1 and H2) in trial 1. Bacterial community structure is significantly different between days 0-1 to days 4-5 and also between days 2-3 to days 4-5.

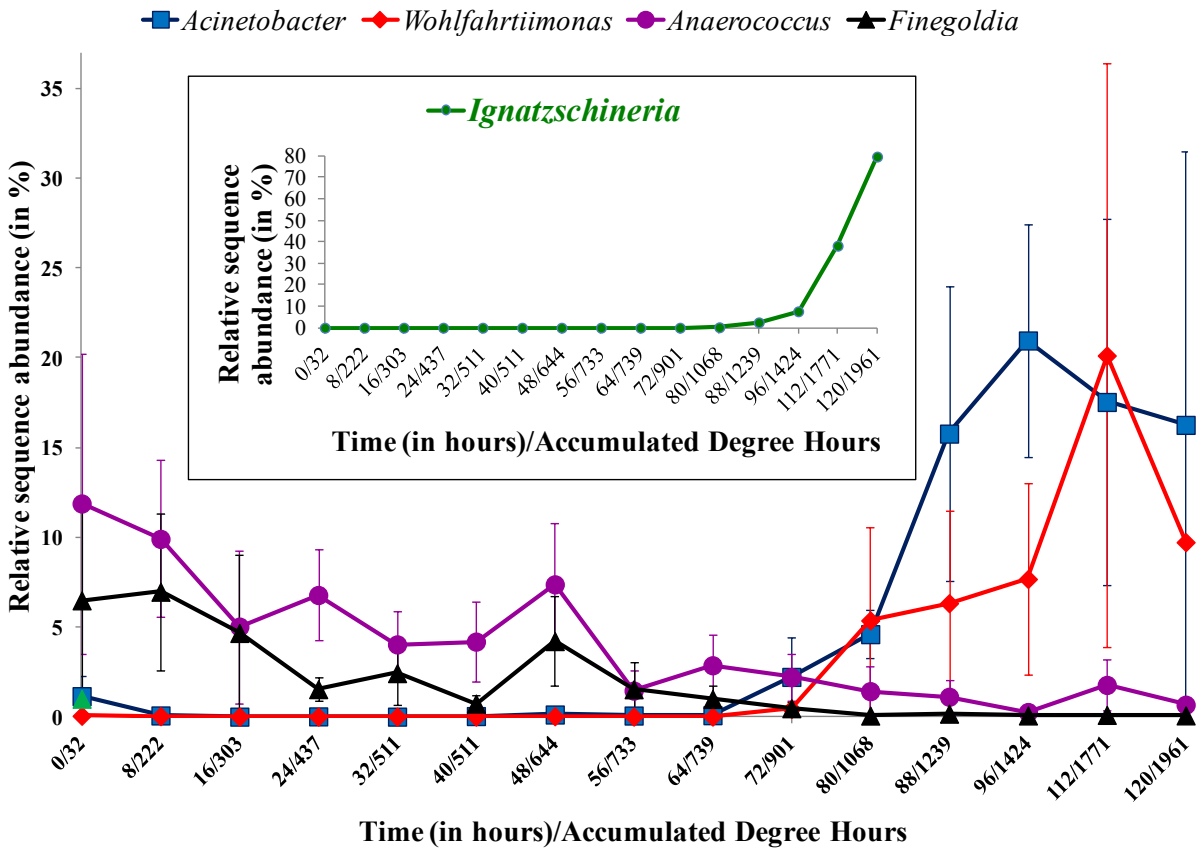


Figure 23. Line graph showing relative sequence abundance of top five indicator genera associated with human cadaver with insect access (ACC) based on pyrosequencing data. Zero degree C was used as a base temperature for calculation of accumulated degree hours (ADH). Error bar indicates standard error of mean (SEM). *Ignatzschineria* was present predominantly in buccal samples.

IV. CONCLUSIONS

We found that there was a significant effect of trial (time of year) on metabolic profiles of humans and that there were only significant changes in these profiles over decomposition or among body sample locations for trial one, but not trials two or three. There were similar findings when human and swine remains were evaluated, with the only significant difference in profiles between species detected during the third trial. It was quite surprising to find very few metabolic profile differences in the field trials or between treatments. This may be due to the low humidity conditions of the study site where many of the human remains mummify quickly in the heat or to low statistical power to detect multivariate differences with unavoidably few sets of human and swine remains. Additional analyses of assessing individual carbon sources, rather than entire metabolic profiles, are on-going to increase the statistical power of these tests. Documenting and identifying differences in microbial community function is key to advancing knowledge of the carrion necrobiome and its applicability in forensic science. As far as structure, we determined that bacterial communities could be used in part to determine the amount of time to elapse since the placement of the remains in the field. Furthermore, we were able to determine that insects impacted microbial communities and further research should be conducted to determine the key bacteria species present in the presence or absence of insects. Doing so, would enable researchers to develop simple kits to predict time of death based on the specific bacteria and their population structure over time.

We also were able to develop the sterile techniques to work with bacteria associated with insects in the laboratory. We were able to describe the microbial shifts associated with the presence of fly larvae on a resource. Furthermore, we demonstrated that bacteria associated with decomposing material regulate arthropod attraction and colonization. These data are crucial for explaining decomposition as related to estimating the min-PMI. We have now identified key microbes associated with the decomposition process and continue conduct research examining the role of bacteria in arthropod development.

Implications for policy and practice. Current policy needs to consider the nature of the data being produced with regards to forensic microbiology and its applications in criminal investigations. The amount of data produced from living or dead humans is substantial and current infrastructure associated with crime laboratories most likely is not adequate to appropriately support new and possibly transformative methodologies like those tested in this research. Policy needs to focus on providing the necessary funds to truly investigate and expand the application of microbiology in forensics. The funding currently available to the National Institute of Justices is allowing researchers to "scratch" the surface of questions related to forensics. We urge Congress to consider increasing funds so that more in depth studies can be conducted ensuring the criteria established by the National Research Council in 2009 are met and a true appreciation of the accuracy of such information is known and applied in our society.

Implications for further research. Results from our research indicate there is still quite a bit to be accomplished prior to implementation of such science in a forensic setting. Research is needed to determine the level of geographic variability associated with the human necrobiome and how abiotic factors regulate the shifts in the necrobiome over time. Furthermore, research is

needed to validate these procedures and determine the level of accuracy presented from these techniques. In the future, crime laboratories will be faced with more sophisticated, yet highly reliable, methodologies associated with microbiology like the ones developed in this research; however, additional funding and support is necessary to investigate the variability from crime scene to crime scene and how to effectively implement such approaches like high throughput technologies into routine forensic use.

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VI. DISSEMINATION OF RESEARCH FINDINGS

Accomplishment #11: Publication of research.

1. **Pechal, J.L., T.L. Crippen, M.E. Benbow, A.M. Tarone, S. Dowd, and J.K. Tomberlin. 2013.** A new forensic tool: using the necrobiome to estimate the PMI_{min}. International Journal of Legal Medicine. DOI 10.1007/s00414-013-0872-1

2. **Zheng, L., T.L. Crippen, L. Holmes, B. Singh, M.L. Pimsler, M.E. Benbow, A.M. Tarone, S. Dowd, Z. Yu, S. VanLaerhoven, T.K. Wood, and J.K. Tomberlin. 2013.** Bacteria mediate oviposition by the black soldier fly, *Hermetia illucens* (L.), (Diptera: Stratiomyidae). NATURE Scientific Reports. DOI: 10.1038/srep02563

3. **Tomberlin, J. K., T. L. Crippen, A. M. Tarone, B. Singh, K. Adams, Y. H. Rezenom, M. E. Benbow, M. Flores, M. Longnecker, J. L. Pechal, D. H. Russell, R. C. Beier, and T. K. Wood. 2012.** Interkingdom response of flies to bacteria mediated by fly physiology and bacterial quorum sensing. Animal Behaviour 84: 1449-1456.

4. **Zheng, L., T. L. Crippen, B. Singh, A. M. Tarone, S. Dowd, Z. Yu, T. K. Wood, and J. K. Tomberlin. 2013.** Bacterial diversity from successive life stages of black soldier fly (Diptera: Stratiomyidae) using 16S rDNA pyrosequencing. Journal of Medical Entomology 50: 647-658.

5. **Davis, T.S., T.L. Crippen, R.W. Hofstetter, and J.K. Tomberlin. *Invited* 2013.** Microbial volatile emissions as arthropod semiochemicals. Journal of Chemical Ecology 39: 840-859.

6. **Pechal, J.L., A. Lewis, J.K. Tomberlin, T.L. Crippen, A.M.Tarone, and M.E. Benbow. *Accepted* 2013.** Metabolic profiles of carrion decomposition. *PLoS ONE.*

7. **Pechal, J.L., M.E. Benbow, T.L. Crippen, A.M. Tarone, and J.K. Tomberlin. *Resubmitted* 2013.** Delayed access alters necrophagous insect community assembly and carrion decomposition. Oecologia (resubmission under review).

8. **M.J. Scott, M.L. Pimsler, A.M. Tarone. *Invited Submission 2013.*** Sex determination mechanisms in calliphorids (blow flies). Sexual Development.

Accomplishment #12: Presentations of research.

1. **Pechal, JL, H Moore, F Drijfout, and ME Benbow. 2013.** Hydrocarbon Profile Changes Throughout Adult Calliphoridae Aging. 11th North American Forensic Entomology Association Annual Meeting, Dayton, OH, 15-17 July.

2. **McHugh*, M, A Whitaker*, JL Pechal, and ME Benbow. 2013.** Calliphoridae Diversity in Appalachian Ecoregions. 11th North American Forensic Entomology Association Annual Meeting, Dayton, OH, 15-17 July.

3. **Benbow, M.E.** Bugs and bodies: new frontiers and dimensions of forensic entomology. Invited Presentation for the 32nd Annual Empire State Association of Two Year College Biologists, Albany, New York, Apr 20, 2013.

4. **Benbow, M.E.** Carrion ecology, evolution and their applications: new insights into microbe-insect interactions of ephemeral resources. Invited Graduate Luncheon Seminar in the Department of Integrative Biology, University of California Berkeley, Apr 15, 2013.

5. **Benbow, M.E.** Insects and incarceration - maggots, medicine and microbes, oh my! Invited Seminar in the Division of Arts and Sciences, Kettering College, Feb 14, 2013.

6. **Tomberlin, J.K. 2013.** Quorum sensing by bacteria regulates interkingdom interactions on vertebrate carrion: a new frontier for forensics. School of Agricultural, Forest, and Environmental Sciences, Clemson University.

7. **Tomberlin, J.K. 2013.** Bridging entomology and microbiology: a new frontier in forensics. UNICAMP, Universidade Estadual de Campinas.

8. **Tomberlin, J.K. 2013.** New frontiers in forensic entomology. Forensic Sciences and Criminal Behaviour Conference, Instituto Superior de Ciencias da Saude Egas Moniz, Lisbon, Portugal.

9. **Tarone, A.M.** High-throughput Sequencing to Identify Sex Determining Genes in *Chrysomya rufifacies* Macquart (Diptera: Calliphoridae). Second Annual Whole Systems Genomics Initiative Symposium.

10. **Tarone, A.M.** George Bush Library, Texas A&M University, September 19, 2012. The genomics of blow fly development: Advancing research in forensic science and sex determination. Texas Genetics Society. College Station, TX, May 2013.

11. **Tarone, A.M.** Proper methods for collecting insects from a crime scene. TEEX Skeletal Death Investigation Course. Forensic Anthropology Center, Texas State University, San Marcos, TX. May 21, 2013.

12. **Tomberlin, J.K. 2013.** New frontiers in forensic entomology. Forensic Sciences and Criminal Behaviour. Instituto Superior de Ciencias da Saude Egas Moniz. Lisbon, Portugal.

13. **Pechal, J.L., M.E. Benbow, T.L. Crippen, A.M. Tarone, and J.K. Tomberlin. 2013.** Forensic Ecology: Using insects and bacteria for criminal investigations. 32nd Empire State Association of Two Year College Biologists Annual Meeting. Cobleskill, NY, 19-21 April 2013. (*Invited presentation).*

14. **Pimsler, M.L., S.H. Sze, J.K. Tomberlin, and A.M. Tarone. 2013.** Analysis of the *de novo* transcriptome of Immature *Chrysomya rufifacies* (Diptera: Calliphoridae) to investigate sexually dimorphic patterns of gene expression and their role in m-PMI estimates with a forensically important fly. American Academy of Forensic Sciences. Washington, DC.

15. **Pimsler, M.L., S.-H. Sze, C.D. Jones, J.K. Tomberlin, A.M. Tarone.** Elucidation of the sex-determination pathway in a blow fly with monogenic sex determination (Poster). Drosophila Research Conference, Washington DC, April 2013.

16. **Pimsler, M.L., S.-H. Sze, J.K. Tomberlin, and A.M. Tarone.** Temporally and sexually dimorphic patterns of gene expression from a *de novo* transcriptome in an invasive fly. Ecological Integration Symposium, College Station, TX, April 2013.

17. **Benbow, M.E., J.K. Tomberlin, T.L. Crippen, A.M. Tarone, T. Wood, H.N. LeBlanc, J.L. Pechal. 2012.** Microbes, maggots and multiplicity: Biotic and abiotic complexities of carrion decomposition. 60th Entomological Society of America Annual Meeting, Knoxville TN, 11-14 November.

18. **Flores, M., J. Rhinesmith, T. Crippen, T. Wood, A. Tarone, and J.K. Tomberlin. 2012.** Quorum sensing by *Escherichia coli* serves as interkingdom signal with *Lucilia sericata* (Diptera: Calliphoridae). 10th Annual North American Forensic Entomology Conference, Las Vegas, Nevada.

19. **Pechal, J.L., M.E. Benbow, T.L. Crippen, A.M. Tarone, and J.K. Tomberlin. 2012.** Bacteria and blow fly interactions throughout vertebrate decomposition. 60th Entomological Society of America Annual Meeting, Knoxville TN, 11-14 November. (*Invited oral presentation)*

20. **Pechal, J.L., M.E. Benbow, T.L. Crippen, A.M. Tarone, and J.K. Tomberlin. 2012.** Bacteria communities predicting insect composition on an ephemeral resource. 60th Entomological Society of America Annual Meeting, Knoxville TN, 11-14 November.

21. **Pechal, J.L., M.E. Benbow, T.L. Crippen, A.M. Tarone, and J.K. Tomberlin.** 2012. Decomposers of carrion: Structural and functional relationships of micro- and

macroorganisms removing vertebrate carcasses from terrestrial ecosystems. 24th International Congress of Entomology Meeting, Daegu, South Korea, 20-25 August.

22. **Pechal, J.L., M.E. Benbow, T.L. Crippen, A.M. Tarone, and J.K. Tomberlin. 2012.** Quantifying microbe-insect interactions to predict minimum postmortem intervals. 10th North American Forensic Entomology Association Annual Meeting, Las Vegas, NV, 17-20 July.

23. **Pechal, J.L., M.E. Benbow, T.L. Crippen, A.M. Tarone, and J.K. Tomberlin. 2011.** Community composition and assembly on decomposing vertebrate carcasses using pyrosequencing. 59th Entomological Society of America Annual Meeting, Reno, NV, 13-16 November.

24. **Pechal, J.L., M.E. Benbow, and J.K. Tomberlin. 2011.** Invertebrate community successional changes resulting from delayed colonization on ephemeral resources. 59th Entomological Society of America Annual Meeting, Reno, NV, 13-16 November.

25. **Singh, B., A.M. Tarone, T. Crippen, M.E. Benbow, L. Zheng, Z. Yu, A. Fields, M. Flores, S. Dowd, T. Wood, and J.K. Tomberlin. 2012.** Bacteria diversity associated with *Hermetia illucens* (Diptera: Stratiomyidae), *Lucilia sericata* and *Lucilia cuprina* (Diptera: Calliphoridae). 10th Annual North American Forensic Entomology Conference, Las Vegas, Nevada.

26. **Pechal, J.L., M.E. Benbow, T.L. Crippen, A.M. Tarone and J.K. Tomberlin. 2012.** Utilizing bacterial community succession to predict the postmortem interval of a corpse: implications for forensic entomology. 9th European Association for Forensic Entomology Annual Meeting, Toruń, Poland, 18-21 April.

27. **Tarone, A.M.** CSI: Dipteran Genomics. University of Dayton, March 8, 2012.

28. **Tarone, A.M.** CSI: Dipteran Genomics. Texas A&M University, Freshman Honors Housing Community, Lechner Hall, April 16, 2012.

29. **Picard, C.J., K. DeBlois, F. Tovar, J.S. Johnston, A. Tarone.** Applications of Genome Sizes in Forensic Entomology. European Association of Forensic Entomology, 9th Annual Meeting, Torun, Poland, April 18-21 April 2012.

30. **Tomberlin, J.K. 2012.** Behavioral echoes of blow flies (Diptera: Calliphoridae) associated with resource pulses. Department of Biology, St. Edwards University, Austin, Texas.

31. **Tomberlin, J.K. 2012.** Interkingdom eavesdropping explains arthropod behavior. Ecolunch, Section of Integrative Biology, University of Texas.

32. **Tomberlin, J.K. 2012.** Analysis of behavioral data. 2012 Probability and Statistics Day. Department of Statistics, Texas A&M University.

33. **Benbow, M.E., J.L. Pechal and J.K. Tomberlin. 2012.** Community succession effects of delaying necrophagous insect colonization of carrion. 9th European Association for Forensic Entomology Annual Meeting, Toruń, Poland, 18-21 April. *(abstract)*.

34. **Diaz, M., A. Sreenivasan, M.E. Benbow. 2012.** The metabolic diversity of blow fly (*Lucilia sericata*) associated bacteria. Emerging Researchers National Conference in Science, Technology, Engineering, and Mathematics (STEM), 23-25 February 2012. Atlanta, GA.

35. **Benbow, M.E., A Lewis. 2011.** Necrophagous invertebrate community assembly in relation to microbial metabolic activity on a carrion resource: exploring ecological mechanisms of vertebrate decomposition. Annual Meeting of the Entomological Society of America, 13-16 November 2011. Reno, NV.

36. **Pechal, J.L., Benbow, M.E., T.L. Crippen, A.M. Tarone, J.K. Tomberlin. 2011.** Community composition and assembly on decomposing vertebrate carcasses using pyrosequencing. Annual Meeting of the Entomological Society of America, 13-16 November 2011. Reno, NV. (*Invited Oral Presentation*).

37. **Pechal, J.L., Benbow, M.E., T.L. Crippen, A.M. Tarone, J.K. Tomberlin. 2011.** Invertebrate community succession changes resulting from delayed colonization on ephemeral resources, 13-16 November 2011. Reno, NV.

38. **Pechal, J.L., M.E. Benbow, T.L. Crippen, A.M. Tarone, J.K. Tomberlin. 2011.** Microbial community function on decomposing vertebrate carrion. Bull. Eco. Soc. Am. 20th Annual Meeting Abstracts, 7-12 August 2011. Austin, TX. (*abstract*).

39. **Pechal, J.L., M.E. Benbow, T.L. Crippen, A.M. Tarone, J.K. Tomberlin. 2011.** A novel approach in forensic estimations of period of insect activity: microbial community profiles as indicators of decomposition. 9th Annual Meeting of North American Forensic Entomology Association, College Station, TX, 21-23 July 2011. *(abstract)*.

40. **Pechal, J.L., M.E. Benbow, T.L. Crippen, A.M. Tarone, J.K. Tomberlin. 2011.** A novel approach in forensic estimations of period of insect activity: microbial community profiles as indicators of decomposition. First Annual Meeting of the Malaysia Association of Forensic Entomology. 6 July 2011, Kuala Lumpur, Malaysia. *(Invited Oral Presentation)*.

41. **Benbow, M.E., J.K. Tomberlin, A.M. Tarone, T.L. Crippen, T.K. Wood, H. LeBlanc. 2011.** Keynote: Understanding the mechanistic role of blow flies in the microbial ecology of carrion decomposition: implications to food borne disease spread. One Day Seminar "Forensic Entomology and It's Implications in Medicine". Universiti Teknologi Mara, Kuala Lumpur, Malaysia, 5 July 2011. (*Invited Keynote Speaker*).

42. **Benbow, M.E., J.K. Tomberlin, A.M. Tarone, T.L. Crippen, T.K. Wood, H. LeBlanc. 2011.** New approaches for understanding the mechanistic role of microbial community-blow fly interactions during carrion decomposition: applications to forensic science. International Conference on Forensic Entomology. Guangdong Police College, Guangzhou, China, June 28, 2011. *(Invited Oral Presentation)*.

43. **Tomberlin, J.K., M.E. Benbow, T. Crippen, A. Tarone, H. LeBlanc, and T. Wood. 2011.** Keynote: Forensic entomology: past, present and future. Universiti Teknologi Mara, Kuala Lumpur, Malaysia.

44. **Tomberlin, J.K. 2011.** Keynote: Case studies in forensic entomology. Universiti Teknologi Mara, Kuala Lumpur, Malaysia.

45. **Benbow, M.E., J.K. Tomberlin, J.L. Pechal, T. Crippen, A.M. Tarone, T. Wood, A. Lewis. July 2011.** Understanding the mechanistic role of blow flies in the microbial ecology of carrion decomposition: implications to food borne disease spread. Seminar, Faculty of Medicine, University Teknologi Mara, Malaysia.

46. **Tomberlin, J.K., M.E. Benbow, T.L. Crippen, A.M. Tarone, H. LeBlanc, T.K. Wood.** Microbial puppet masters of blow fly behavior. State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, Wuhan, China, June 24, 2011. *(Invited Oral Presentation)*.

47. **Benbow, M.E., J.K. Tomberlin, A.M. Tarone, T.L. Crippen, T.K. Wood, H. LeBlanc. 2011.** Blow flies, bacteria and inter-kingdom ecological interactions during decomposition: applications for forensic science and beyond. Global Conference on Entomology, Chiang Mai, Thailand, 5-9 March 2011. (*Invited Keynote Speaker*).

48. **Tomberlin, J.K. 2011.** Forensic entomology. Forensic Sciences, St. Edwards University, Austin, Texas.

49. **Tomberlin, J.K. 2011.** Behavioral echoes of saprophytic dipteran ecology- how is that related to forensics? Department of Biology, University of Texas, Tyler, Texas.

50. **Tomberlin, J.K. 2011.** Forensic entomology for writers. Brazos Writers Association. College Station, Texas.

51. **Tomberlin, J.K., M.E. Benbow, T. Crippen, A. Tarone, H. LeBlanc, and T. Wood. 2011.** Forensic entomology: past, present and future. International Conference on Forensic Entomology, Guangzhou Police College, Guangzhou, China.

52. **Tomberlin, J.K. 2011.** An update on black soldier fly research. International Conference on Forensic Entomology, Guangzhou Police College, Guangzhou, China.

APPENDICES

Appendix 1

Int J Legal Med
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ORIGINAL ARTICLE

The potential use of bacterial community succession in forensics as described by high throughput metagenomic sequencing

Jennifer L. Pechal · Tawni L. Crippen · M. Eric Benbow · Aaron M. Tarone · Scot Dowd · Jeffery K. Tomberlin

Received: 11 March 2013 / Accepted: 7 May 2013

Abstract Decomposition studies of vertebrate remains primarily focus on data that can be seen with the naked eye, such as arthropod or vertebrate scavenger activity, with little regard for what might be occurring with the microorganism community. Here, we discuss the necrobiome, or community of organisms associated with the decomposition of remains, specifically, the "epinecrotic" bacterial community succession throughout decomposition of vertebrate carrion. Pyrosequencing was used to (1) detect and identify bacterial community abundance patterns that described discrete time points of the decomposition process and (2) identify bacterial taxa important for estimating physiological time, a time–temperature metric that is often commensurate with minimum post-mortem interval estimates, via thermal summation models. There were significant bacterial community structure differences in taxon richness and relative abundance patterns through the decomposition process at both phylum and family taxonomic classification levels. We found a significant negative linear relationship for overall phylum and family taxon richness as decomposition progressed. Additionally, we developed a statistical model using high throughput sequencing data of epinecrotic bacterial communities on vertebrate remains that explained 94.4 % of the time since placement of remains in the field, which was within 2–3 h of death. These bacteria taxa are potentially useful for estimating the minimum post-mortem interval. Lastly, we provide a new framework and standard operating procedure of how this novel approach of using

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Appendix 2

Plos One

Microbial Community Functional Change During Vertebrate Carrion Decomposition

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Appendix 3

THE IMPORTANCE OF MICROBIAL AND PRIMARY COLONIZER

INTERACTIONS ON AN EPHEMERAL RESOURCE

A Dissertation

by

JENNIFER LYNNE PECHAL

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

DOCTOR OF PHILOSOPHY

May 2012

Major Subject: Entomology

Appendix 4

Analysis of the Volatile Organic Compounds Produced by the Decomposition of Pig Carcasses and Human Remains

by

Sonja Stadler

A Thesis Submitted in Partial Fulfillment
of the Requirements for the Degree of

Doctor of Philosophy in Applied Bioscience

In

The Faculty of Science

Applied Bioscience

University of Ontario Institute of Technology

March 2013

Appendix 5

FITNESS EFFECTS OF COLONIZATION TIME OF *CHRYSOMYA RUFIFACIES* AND *COCHLIOMYIA MACELLARIA,* AND THEIR RESPONSE TO INTRA- AND INTER-SPECIFIC EGGS AND EGG-ASSOCIATED MICROBES

A Dissertation

by

ADRIENNE LEANE BRUNDAGE

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

DOCTOR OF PHILOSOPHY

Approved by:

Chair of Committee,	Jeffery K. Tomberlin
Committee Members,	Micky Eubanks
	Tawni L. Crippen
	Craig J. Coates
	Michael Manson
Head of Department,	David Ragsdale

May 2012

Major Subject: Entomology

Appendix 6

A Survey of Bacterial Diversity From Successive Life Stages of Black Soldier Fly (Diptera: Stratiomyidae) by using 16S rDNA Pyrosequencing

Author(s): Longyu Zheng , Tawni L. Crippen , Baneshwar Singh , Aaron M. Tarone , Scot Dowd , Ziniu Yu , Thomas K. Wood , and Jeffery K. Tomberlin
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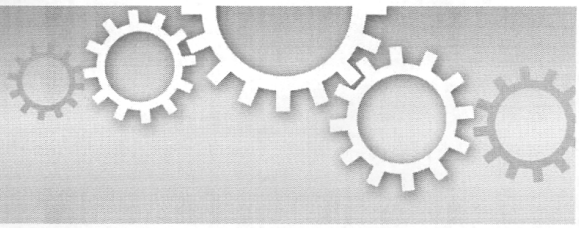
Appendix 7

SCIENTIFIC REPORTS



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Bacteria Mediate Oviposition by the Black Soldier Fly, *Hermetia illucens* (L.), (Diptera: Stratiomyidae)

Longyu Zheng[1,4], Tawni L. Crippen[2], Leslie Holmes[3]*, Baneshwar Singh[4]†, Meaghan L. Pimsler[4], M. Eric Benbow[5], Aaron M. Tarone[4], Scot Dowd[6], Ziniu Yu[1], Sherah L. Vanlaerhoven[3], Thomas K. Wood[7] & Jeffery K. Tomberlin[4]

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There can be substantial negative consequences for insects colonizing a resource in the presence of competitors. We hypothesized that bacteria, associated with an oviposition resource and the insect eggs deposited on that resource, serve as a mechanism regulating subsequent insect attraction, colonization, and potentially succession of insect species. We isolated and identified bacterial species associated with insects associated with vertebrate carrion and used these bacteria to measure their influence on the oviposition preference of adult black soldier flies which utilizes animal carcasses and is an important species in waste management and forensics. We also ascertained that utilizing a mixture of bacteria, rather than a single species, differentially influenced behavioral responses of the flies, as did bacterial concentration and the species of fly from which the bacteria originated. These studies provide insight into interkingdom interactions commonly occurring during decomposition, but not commonly studied.

Interactions between microbes and multicellular organisms are often challenging to characterize. No other place is this more apparent than in systems where there is competition for ephemeral resources. Janzen[1] proposed that single-celled organisms on decomposing materials, such as seed, fruits and even carrion, function as more than simple nutrient recyclers. They are in fact members of the complex community competing for these resources, and through evolutionary time, have developed strategies for reducing competition with prokaryote and eukaryote consumers. However, it took 30 years before Janzen's concept was validated when Burkepile et al.[2] reported that fish carrion contaminated with fewer bacteria were attractive to scavengers for a much longer period of time, and to a wider array of scavengers, than those with uninhibited bacterial fauna. These results indicated bacterial activity reduced competition with scavengers for the resource[2]. However, this effect did not apply to all competitors as some scavengers were actually more successful on the bacteria laden resource[2].

Microbes have long been recognized for their functional importance in driving colonization of a resource by arthropods. Holdaway[3] and Seddon[4] proposed that ammonia produced by bacterial putrefaction on sheep stimulated oviposition by blow flies (Diptera: Calliphoridae). In comparison, gravid mosquitoes, *Aedes aegypti* (L.) (Diptera: Culicidae), must locate water sources that exhibit the appropriate environmental conditions for the development of their offspring[5]. A primary factor regulating attraction and colonization of these sites by female mosquitos is the associated microbial flora[6], where it is the specific combination of 14 bacterial species responsible for the attraction[5]. Gravid house flies, *Musca domestica* L. (Diptera: Muscidae) evaluate volatiles produced by microbes on conspecific eggs to ensure synchronous larval development which allows for aggregative feeding and reduced likelihood of cannibalism[7]. Bacteria associated with these eggs also provided initial food resources[8] and protection from pathogenic fungi on carrion[9].

Recent advances in technology have expanded the tools available for the study of microbial ecology. Limitations existed in the ability to recover most community bacteria via conventional culture-based methods, thus providing gross under-estimates of microbial diversity in nature. Some anaerobic or microoxic bacteria require specific nutrients, or interactions with other organisms to grow and reproduce; factors that have made it

Appendix 8

LIFE-HISTORY TRAITS OF *CHRYSOMYA RUFIFACIES* (MACQUART) (DIPTERA: CALLIPHORIDAE) AND ITS ASSOCIATED NON-CONSUMPTIVE EFFECTS ON *COCHLIOMYIA MACELLARIA* (FABRICIUS) (DIPTERA: CALLIPHORIDAE) BEHAVIOR AND DEVELOPMENT

A Dissertation

by

MICAH FLORES

Submitted to the Office of Graduate Studies of
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in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Approved by:

Chair of Committee,	Jeffery K. Tomberlin
Committee Members,	S. Bradleigh Vinson
	Aaron M. Tarone
	Michael Longnecker
Head of Department,	David Ragsdale

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Appendix 9

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Interkingdom responses of flies to bacteria mediated by fly physiology and bacterial quorum sensing

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Insect location and utilization of a resource is influenced by a host of variables including nutrients acquired prior to encountering a stimulus and age of the individual. For the carrion system, we hypothesized that volatiles to which primary colonizers, such as blow flies, respond are the same signalling molecules produced and utilized for quorum sensing by bacteria found on the resource. We provided freshly emerged blow flies, *Lucilia sericata*, different diets (blood or powdered milk) and assessed their behaviour in a dual-choice assay based on sex and ovarian status of 7-day-old or 14-day-old adults. We determined their preference between wild-type *Proteus mirabilis*, which is able to swarm (a quorum-sensing response), or mutated (by transposon mutagenesis) *P. mirabilis*, which is unable to swarm. In most instances, an individual's sex did not significantly influence its response. Age and diet appeared to regulate fly motivation and preference. Seven-day-old flies had a significantly greater probability of responding to the wild type than to the mutant, regardless of diet, but the percentage of milk-fed flies that responded was significantly smaller (85% less) than the percentage of blood-fed flies that responded. Blood-fed flies oviposited, whereas milk-fed flies did not. Seven-day-old flies oviposited predominately on the wild type, whereas 14-day-old flies oviposited predominately on the mutant. Our results demonstrate that the mechanism used by *L. sericata* for detecting a resource can be associated with bacterial quorum sensing, and that the physiological state of the insect influences its response. We also identified several differences in volatile compounds produced by the bacteria that could explain blow fly response.

The unpredictable occurrence of carrion within an ecosystem results in intense competition for these ephemeral resources. With regard to Diptera, arrival out of sequence in the decomposition process could be detrimental to the resulting offspring (Shorrocks & Bingley 1994; Lam et al. 2007) and their ability to locate a suitable mate (Norris 1965; Archer & Elgar 2003). Consequently, arthropods that utilize these resources have evolved highly sensitive sensory systems allowing for the quick discovery, colonization and utilization of these ephemeral resources (Dethier 1947; Spivak et al. 1991).

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Bacteria associated with carrion release volatile organic compounds (VOCs) that mediate attraction and oviposition by blow flies (Diptera: Calliphoridae) (LeBlanc 2008). In many instances, specific bacteria are responsible for attracting myiasis-causing blow flies to wounds (Khoga et al. 2002). Bovine blood inoculated with one of eight bacteria species, or in combination, was examined for eliciting attraction and oviposition of *Cochliomyia hominivorax* Coquerel (Diptera: Calliphoridae) (Chaudhury et al. 2010). All bacteria species elicited a response by the blow fly; however, *Proteus mirabilis* incubated in blood for 24 h was one of only two species to elicit an oviposition response.

Many of the VOCs associated with carrion decomposition have been surveyed (LeBlanc 2008). And, in some instances, the VOCs identified, such as indole, sodium sulfide (Urech et al. 2004) and

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Appendix 10

Microbial Volatile Emissions as Insect Semiochemicals

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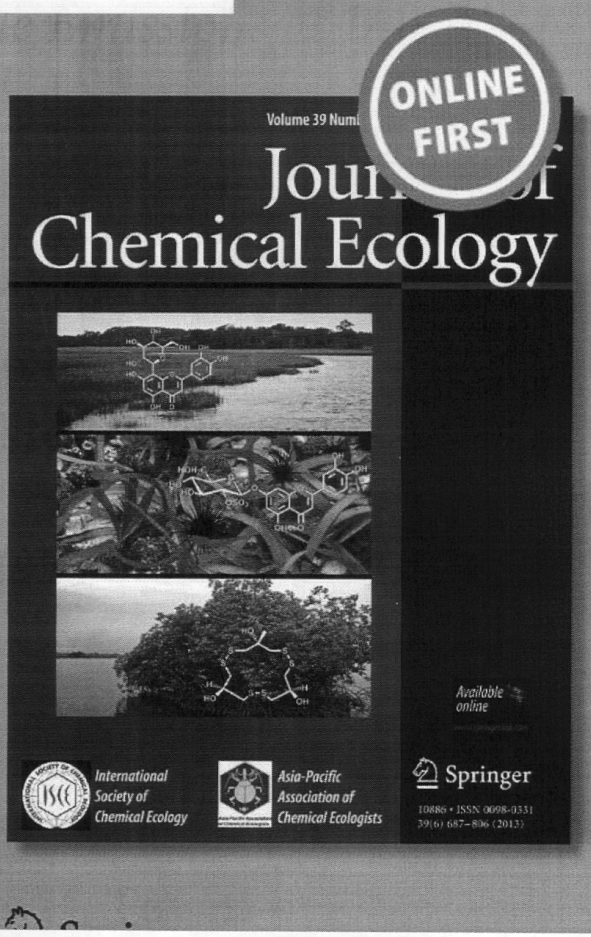

Appendix 11

Submitted to Oecologia

Running Head: *Necrophagous insect assembly and carrion decomposition*

Delayed insect access alters carrion decomposition and necrophagous insect community assembly

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Author Contributions: JLP, MEB, TLC, AMT, JKT conceived and designed the experiments.

Appendix 12

Sex Determination Mechanisms in Calliphorids (Blow flies)

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Abstract

The Calliphoridae or blow flies are a family of insects that occupy diverse habitats and perform important ecological roles, particularly the decomposition of animal remains. Some Calliphoridae species are also important in the forensic sciences, in agriculture (e.g. as livestock pests) and in medicine (e.g. maggot therapy). Calliphoridae provide striking examples in support of Wilkins' hypothesis that sex determination regulatory gene hierarchies evolve in the reverse order [Wilkins, 1995], with the gene at the top being the most recently added. Unlike the model fly *Drosophila melanogaster*, where sex is determined by the number of X chromosomes, in the Australian sheep blow fly (*Lucilia cuprina*) sex is determined by a Y-linked male determining gene (*M*). A different regulatory system appears to operate in the hairy maggot blow fly (*Chrysomya rufifacies*) where the maternal genotype determines sex. It is hypothesized that females heterozygous for a dominant female determining factor (*F/f*) produce only female offspring and homozygous *f* females produce only sons. The bottom of the regulatory hierarchy appears to be the same in *D. melanogaster* and *L. cuprina*, with sex-specific splicing of *doublesex* transcripts being controlled by the female-specific Transformer (TRA) protein. We discuss a model that has been proposed for how *tra* transcripts are sex-specifically spliced in calliphorids, which is very different than from *D. melanogaster*.