THE IMPACT OF MICROBES ASSOCIATED WITH VERTEBRATE CARRION ON ATTRACTION AND COLONIZATION BY *COCHLIOMYIA MACELLARIA* (FABRICIUS) (DIPTERA: CALLIPHORIDAE)

A Dissertation

by

CASEY ANN FLINT

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Chair of Committee,	Jeffery K. Tomberlin
Committee Members,	Tawni L. Crippen
	Anjel Helms
	Adrienne Brundage
	Aaron Tarone
Head of Department,	Phillip Kaufman

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ABSTRACT

Biotic and abiotic factors that affect insect colonization of a carrion resource have been vastly studied. Such information is used to inform the time of colonization or post-colonization interval, which is crucial for determining the time since death (i.e., postmortem interval [PMI]). However, little is known about the period in which insects detect, locate, and assess the carrion resource (pre-colonization interval [pre-CI]). A key factor suspected to play a crucial role in regulating such responses during the pre-CI, but also relatively under-studied, is the microbiome of the host at the time of death. Microbes are known to produce volatile organic compounds (VOCs), which flies use as cues to locate these resources. Deciphering the factors regulating insect attraction and colonization could provide valuable data leading to accurate determination of the true PMI. A dual-choice cube olfactometer was tested for use with adult *Cochliomyia macellaria* as a study species which provided optimal parameters to use during future experiments related to adjustment time, trial length, sugar and water presence or absence, and mesh type used in the olfactometer. To investigate the impact of the carrion microbiome on insect attraction, responses of adult male, gravid and non-gravid *Cochliomyia macellaria* to xenic (i.e., with a microbiome) and axenic (i.e., without a microbiome) mice were determined. Adults significantly preferred the xenic, rather than the axenic, mouse by an overall average of 15% with the largest difference on day 4 (40% difference). In conjunction with the behavioral assessment of the blow fly, VOC profiles were also assessed to determine which compounds were produced by each mouse

treatment. Each treatment produced a distinct VOC profile, and seven indicator compounds were identified for the xenic mouse with six being microbially derived. In addition to these findings, an oviposition assay was conducted with the treatments resulting in a ~90% reduction in oviposition without microbial presence on the carrion resource. Results indicate microbial presence on a carrion resource is an important factor for blow fly attraction and acceptance of a carrion resource for reproduction. Data provide an important foundation for further research on insect-microbe interactions in decomposition related to the pre-CI.

DEDICATION

This dissertation is dedicated to my father, Gary Reece Hearn, who was the inspiration and drive for me to continue my education. Love you more.

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TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

1. INTRODUCTION

Carrion Ecology

The breakdown of detritus is an essential component for nutrient recycling (Moore et al. 2004). Detritus has historically been defined as the reduction of organic carbon (i.e., dead organic matter (Swift et al. 1979)) from any trophic level (includes egestion, excretion, and secretion) by non-predatory means (Wetzel et al. 1972). Detritus can range from low quality dead plant matter to nutrient-rich dead animal tissue (Wilson and Wolkovich 2011). Detritus is mostly carbon, but can be a combination of nitrogen, phosphorus, potassium, and other essential nutrients (Carter et al. 2006, Benbow et al. 2015). A relatively understudied category of detritus, in comparison to plant matter, is vertebrate carrion.

What is Carrion?

Carrion is defined as dead and decaying flesh or the remains of a vertebrate organism (Wilson and Wolkovich 2011), which can include sea life (Britton and Morton 1994), swine (Weatherbee et al. 2017), poultry (Shean et al. 1993), cattle (Towne 2000), and even humans (Catts and Goff 1992) to name a few. Carrion has three defining characteristics that make distinguish it from other food resources; it typically is an ephemeral resource, its occurrence in nature is usually unpredictable, and when available, competition for it is intense. Carrion is an ephemeral resource due to the combination of natural decomposition and other organisms quickly consuming the remains, however there can be exceptions. As the number of consumers increases, the duration of the carrion

resource can be shortened (Payne 1965, Kneidel 1984). For example, carrion available for insect colonization will decompose at a faster rate than carrion that is not available for insect colonization. A pig carcass has been shown to retain approximately 75-80% of the initial body weight five days after placement with no insect activity, while a carcass exposed to insect colonization retained approximately 10% of the initial body weight after the same amount of time (Payne 1965).

There are exceptions to the ephemeral characteristic of carrion, including megafauna deaths and mass mortality events. Megafauna are described as large and giant animals, typically with an adult weight of 450 kg or more. Large whales (27-145 metric tons) can take up to several decades to fully decompose due to decomposer population satiation (Smith and Baco 2003). Carcasses of elephants and giraffes in Africa persist at least 13 times longer than carcasses of smaller herbivores such as wildebeests and zebras (Blumenschine 1989), often putrefying before being completely consumed by scavengers (Pereira et al. 2014). Manner of death can also impact the duration of carcass availability, as individuals who are predated on are often mostly consumed by the predator immediately after death, while carcasses from individuals who perish from nutrient or water deficiency can persist for much longer (Houston 1974).

The availability of carrion in a given ecosystem is not consistent under most circumstances. Carrion availability can be dramatically increased by natural, recurring behaviors and natural hazards. An example of a natural, recurring behavior that increases carrion availability is annual migration. Salmon annually migrate back to their spawning site to produce the next generation (Quinn 2005). Spring-run Chinook salmon

(*Oncorhynchus tshawytscha*) have an estimated 10.4-20.9% mortality rate during their annual migration, producing nutrient resources for other organisms in the surrounding ecosystem (Keefer et al. 2017). One of the natural mild hazards that contribute to carrion availability are drought spells. In the Serengeti, a four-month-long dry season occurs every year. Out of the total annual wildebeest deaths, 64% occur in this 4 month-long dry season, with the other 36% of deaths occurring in the remaining eight months of the year (Mduma et al. 1999). These examples demonstrate that carrion availability fluctuates throughout the year.

Stemming from the high nutrient quality of carrion, many species will rapidly make use of the resource, when available. This leads to immense competition for the high-nutrient resource among microbes, invertebrates, and vertebrate scavengers, especially in warm climates (DeVault et al. 2003) due to increased microbial and invertebrate development, reproduction, and abundance. High competition has led to specialized niches in organisms, and most recognizable, carrion flies. For example, six species of blow flies were shown to thrive in different seasons and carrion sizes, inidicating that species with the same feeding preferences cannot coexist (Denno and Cothran 1975).

Consumers of Carrion

Consumers of carrion vary as much as carrion itself does. Most consumers of carrion are scavengers because they do not kill their food source. Scavengers can be broken up into two groups: obligate or facultative scavengers. Obligate scavengers, such as vultures (Ruxton and Houston 2004) or some blow fly (Diptera: Calliphoridae) species (Stevens 2003), solely rely on carrion for survival while facultative scavengers such as

raccoons (Schoonover and Marshall 1951) or rove beetles (Coleoptera: Staphylinidae) (Dubie and Talley 2017) will feed on carrion, but it is not the primary food source (Wilson and Wolkovich 2011). The relationship between scavenging and recycling nutrients is a relatively unexplored topic that could shed light on different functional groups and their associated roles in this system (Wilson and Wolkovich 2011).

Vertebrates

Vertebrate scavenging is more widespread than recognized in most hypotheses (DeVault et al. 2003). Vertebrates will feed on carrion as a primary food source or as a last resort if it is necessary to survive (Mondor et al. 2012, Sanford 2015). A well-known vertebrate taxon evolved to consume carrion are vultures, as previously mentioned. Aerial scavengers, such as vultures, have an advantage over terrestrial scavengers due to the ability to search a greater area in a given amount of time (Ruxton and Houston 2004). Vertebrates such as mice, rats, coyotes, opossums, raccoons, deer and many bird species other than vultures have been documented eating carrion (Mondor et al. 2012, Meckel et al. 2017). Domestic animals, such as cats and dogs, also have been documented consuming carrion when necessary for survival (Sanford 2015). When feeding, vertebrates usually take large chunks of tissue, leaving less for other organisms and increasing the overall decomposition rate via mass decrease. Vertebrate scavengers are important for recycling carrion abandoned by predators back into the environment by consuming it. In contrast to predation, scavenging allows animals to consume other vertebrates without a predation event, which conserves energy in the ecosystem (Wilson and Wolkovich 2011). Scavenging vertebrates can accept multiple prey species that have been rejected or left by

vertebrate hunters, adding more connections and stabilizing the food web (Wilson and Wolkovich 2011). Wilson (Wilson and Wolkovich 2011) estimates that scavenging has been underestimated by approximately 16-fold in food web research, leading to a lack of research in that area. Vertebrate scavenging research is essential to completely understand the role vertebrates play in nutrient recycling in the environment.

Invertebrates

In many cases, arthropods are the first, and most specious, phylum to arrive at vertebrate carrion. For example, a carrion decomposition study in Clemson, South Carolina found 522 species utilizing the targeted carrion (Payne 1965) with the arthropod orders Coleoptera, Diptera, Hymenoptera, and Araneida comprising 78% of the total carrion species present (Payne 1965). Much of the invertebrate and decomposition literature focuses on Diptera and Coleoptera due to the swift arrival of adults after death, and the use of carrion as an oviposition substrate and nutritional source for their offspring. Adult flies in the family Sarcophagidae have been documented on frozen pig carrion within five minutes of exposure, and Calliphoridae within ten minutes (Payne 1965). Adult common carrion beetles (*Thanatophilus sinuatus*) (Fabricius) (Coleoptera: Silphidae) were documented arriving to carrion after 1.4 days of exposure (Matuszewski and Szafałowicz 2013). However, beetle and fly communities vary spatially and temporally impacting their interaction with carrion resources. For example, a statewide survey of Texas discovered that *Cochliomyia macellaria* had been present in all ten Texas ecoregions while *Lucilia silvarum* (Meigen) (Diptera: Calliphoridae) had only been present in one ecoregion (Faris 2017). The blow fly *Cochliomyia macellaria* (Fabricius)

(Diptera: Calliphoridae) was discovered at urban and rural sites in central Texas year round, predominantly during late spring, summer, and fall, and were usually the first to oviposit on carrion (Tenorio et al. 2003). Carcass size can also affect the insect community at a carrion resource, as larger carrion resources can accommodate a higher diversity and abundance of insect consumers (Matuszewski et al. 2016). Although species communities on carrion vary spatially and temporally, interactions between insects and a carrion resource have been heavily studied through succession observations and development studies with various species.

Microbes

The third main group of organisms that consume carrion are the organisms included in the microbiome, although most research focuses on bacteria. In humans, it was originally estimated that as an adult, microbes outnumber human cells by approximately 10 to 1 (Luckey 1972, Maynard et al. 2012) but has since been revised to an estimate of 2 to 1 or even as low as 1 to 1 in a 70kg "reference man" (Sender et al. 2016). The human microbiome begins at birth and can be affected by mode of delivery, maternal microbiota, and infant feeding methods and continues to expand and diversify throughout the individual's life (Mohajeri et al. 2018). The location of the microbe inside the human body provides a clue as to what its functional role is. The human gastrointestinal tract has the greatest number of microbes and is dominated by *Bacteroides* species which aids in the digestion and fermentation of carbohydrates (Wexler 2007, Zafar and Saier 2021). *Lactobacillus* species are the most abundant species of vaginal bacteria in women and inhibit other bacteria from binding to epithelial cells by binding to the epithelial cells and

producing lactic acid to lower the pH (Blum 2017, Witkin and Linhares 2017). *Streptococcus* species are found all around the human body but are the most abundant species in the human oral cavity and have a variety of functions including producing hydrogen peroxide in the mouth and inhibiting pathogenic species from colonizing and growing (Huse et al. 2012, Blum 2017, Abranches et al. 2018). The most abundant species of bacteria on the skin changes with the area and type of skin with *Propionibacterium* species being most abundant in sebaceous sites and moist areas (Huse et al. 2012, Blum 2017, Byrd et al. 2018).

Each individual's unique microbiome is regulated by the immune system when alive. Once the organism is no longer living and there is no functioning immune system, the microbes already present in and on the body have immediate access to utilize the remains. The microbes fight one another for the nutrients and space while emitting byproducts, sending cues that are available to other organisms (Janzen 1977). Throughout the decomposition process, specific bacteria such as *Bacteroides* and *Lactobacillus sp.* decrease in a significant ($p < 0.05$) and repeatable manner (Heimesaat et al. 2012, Javan et al. 2016).

Staphylococcus, Candida, Malasseria, Bacillus, and Streptococcus are only some of the bacterial genera present during early decomposition and later decomposition is dominated by genera such as *Pseudomonads*, *Flavobacteria*, and *Cytophaga* (Vass 2001). One inconsistency in decomposition microbiome studies is the lack of consistency on level of taxonomy used to identify the bacteria, as some identify to species and others only go to family or genus. Recent studies have begun to document trends in bacterial growth and

changes in community composition after death and in the absence of insect colonization. In swine carcasses, skin and mouth bacteria followed a consistent and repeatable pattern of change during the first five days of decomposition (Pechal et al. 2014). The order Rhizobiales was found to be the most reliable predictor of a post mortem interval in a study conducted with mice (Metcalf et al. 2013). Although much is known about these microbes individually, it is still unknown exactly what combination of microbes are present in carrion, how they aide in decomposition, how they interact with each other, and how they affect attraction of invertebrate consumers (Mondor et al. 2012).

Detection and Location of Carrion by Flies

Based on the high nutrient quality of carrion, variable duration time of availability, and the unpredictable nature of carrion, competition for this type of resource is relatively high. Consequently, for flies to utilize carrion, they must detect, locate, and colonize carrion before vertebrate or other arthropod competitors can consume the carrion source beyond use. This phenomenon has been described in other systems as a 'priority effect' (Wilbur and Alford 1985). Gravid flies must locate carrion and lay eggs before decomposition and microbial proliferation are too advanced for their offspring to survive or before the resource is colonized by other insects. Locating carrion as quickly as possible is essential for offspring survival and has likely influenced morphological changes and sensory adaptations within species over time. This urgency has led to an enhanced visual and olfactory system in Diptera which enables gravid female blow flies to detect and locate decomposing matter within minutes, even as fast as 23 seconds after exposure (Weidner et al. 2016).

Visual

Adult flies have two types of eyes, simple eyes and compound eyes (Sukontason et al. 2008a). Simple eyes, or ocelli, are used to detect changes in light (Zhou et al. 2016). Flies actually see their surroundings through their compound eyes, making it the most important organ for fly vision (Sukontason et al. 2008b). Each facet, or ommatidia, on the compound eye generates a separate part of a picture of the immediate surrounding environment that is combined into a single useful visual in the brain. The number of facets varies by species and sex (Sukontason et al. 2008b). When searching for food, color has an impact on their decisions to pass up a source or to land and feed. Blow flies can differentiate between colors (Wall and Smith 1996) but there are contradicting results describing their color preference. Female flies use sight to help locate a reliable oviposition substrate that will support their offspring. This could be the explanation for why in some species, such as *Lucilia sericata* (Meigen) (Diptera: Calliphoridae) and *Calliphora vicina* (Robineau-Desvoidy) (Diptera: Calliphoridae), females have more developed sight than males (Aak and Knudsen 2011).

Olfaction

Olfaction is the main part of a necrophilous insect's sensory system used to detect carrion (Benbow et al. 2015). Blow flies have a specialized olfactory system that enables them to receive and analyze chemical information from their environment. Adult blow flies receive most of their information about the outside world through their primary olfactory structure, the antennae. The fly antennae are covered with chemoreceptors that help absorb chemicals, with the numbers varying among different species of

Calliphoridae, Muscidae, and Sarcophagidae (Sukontason et al. 2004). Adult blow flies have aristate antennae containing three main sections, with the last section possessing a protruding hair called the arista (Whitworth 2006). A key characteristic to blow fly species identification is the presence or absence of small hairs on the arista (Whitworth 2006). The little hairs on the antennae, called sensory sensilla, have small pores which allow the odorants into the olfactory system for analysis (Brito et al. 2016). The main odorants blow flies use for finding a suitable carrion resource are volatile organic compounds (VOCs) emitted by or associated with carrion.

Volatile Organic Compounds

A diversity of volatile organic compounds (VOCs) are released from carrion throughout the decomposition process, and many have been previously identified. In fact, more than 475 specific VOCs associated with burial decomposition of humans have been identified (Verheggen et al. 2017). Up to 225 of these VOCs were shown to be present at the same time during pig carcass decomposition (Dekeirsschieter et al. 2012, Verheggen et al. 2017). By 2011, 64 VOCs from vertebrate decomposition had been identified and verified by more than two studies (Paczkowski and Schutz 2011).

The VOCs emitted from decomposing carrion provide cues for other organisms to detect and assess whether the resource is appropriate for their use. The combination of VOCs present and the interpreter determine what message is interpreted (Janzen 1977). These can range from "come on over" to "stay away" and anything in between. The carrion associated VOCs indole, putrescine, phenol, and lactic acid are known blow fly attractants (Ma et al. 2012). There is a relationship between sex/physiological state and dosage of

volatiles that determine fly attraction (Liu et al. 2016). However, volatiles are not just emitted from carrion. Blow flies also emit VOCs that other blow flies interpret as attractive. Brodie et al. (Brodie et al. 2015) discovered that blow flies feeding on liver made the liver more attractive to gravid and non-gravid blow flies than unaltered liver. This discovery gives weight to the hypothesis that the volatiles causing blow fly attraction to a resource can be derived from the salivary glands or guts of other blow flies, not solely due to oviposition (Brodie et al. 2015). VOCs associated with bacteria on house fly, *Musca domestica* (Diptera: Muscidae), eggs have been shown to initially induce oviposition while shifting to inhibit oviposition as the bacteria proliferate (Lam et al. 2007), theoretically due to a change in VOC emission.

How VOCs are Microbially Derived

Bacteria break down organic compounds into inorganic compounds to fuel their metabolism. As a byproduct of this process, microbial volatile organic compounds (mVOCs) are produced. Approximately 50-80% of bacteria produce volatiles and over 350 different volatiles have been identified (Schulz and Dickschat 2007).

When an organism is living, the immune system regulates the microbes, and VOCs, within the body. After the organism dies, microbes can grow and reproduce unregulated, which initiate and aide in the decomposition process. Microbes are in a nutrient rich environment and will produce volatiles that will reflect that (Metcalf et al. 2013). The first step of decomposition is autolysis, when enzymes inside the cell begin to digest it form the inside out, leading to cell rupture and the release of nutrient rich fluid (Vass 2001). This allows anaerobic bacteria to flourish as they are now unregulated. The

gases produced by the metabolic activities of anaerobic bacteria include sulfur dioxide, hydrogen sulfide, carbon dioxide, methane, ammonia (Goff 1993) and are determined by the substrate composition that is being metabolized. For example, sulfur based compounds such as dimethyl disulfide and dimethyl trisulfide are typically produced by the breakdown of the amino acid methionine (Lu et al. 2013) and indole is produced by the breakdown of the amino acid tryptophan (Sasaki-Imamura et al. 2010). The buildup of fluid and gas then cause putrefaction and bloating inside the body. Once the gasses and fluids have stretched the barrier to its maximum, the skin will rupture and purge the liquid and gas, turning the environment from anaerobic to aerobic. As decomposition advances and nutrients diminish, microbes adapt in competition, producing a different composition of volatiles (Metcalf et al. 2013). The VOCs produced by microbes are influenced by the nutrients available to them, giving hints to surrounding organisms about the suitability and quality of the carrion (Korpi et al. 2009). These VOCs are public information to surrounding organisms, who can detect and utilize that information for various purposes.

Blow Flies Using Olfaction to Locate Carrion

As decomposition advances, the VOCs released from decomposition and microbial activity change as well. It has been established that the microbial community inside and on carrion changes enough over the course of decomposition to estimate a post mortem interval within approximately 1-5 days (Metcalf et al. 2013). Minute traces of these decomposition odors can be detected by blow flies in air currents (Mondor et al. 2012). Blow flies detect and process this public information (the VOCs) to locate and assess the resource. Many studies have tested singular and mixtures of chemical attractants

to necrophagous flies but have yielded mixed results as to which chemical compounds are most attractive (Brodie et al. 2014) which could indicate that flies are using a suite of VOCs to assess a resource or attraction is affected by phenotype.

Complete headspace VOC analysis is still a highly unexplored concept regarding assessing what volatiles are produced by decomposing carrion. However, this collection method does not lend itself to answering if the VOCs attractive to flies are microbially derived or from the remains (Brodie et al. 2014). Brodie et al. (Brodie et al. 2014) tested blow fly attraction to incised and intact mice before analyzing the headspace volatiles associated with those mice, but still did not answer the question of whether microbes or the carrion tissue alone produces these volatiles. This research will test whether VOCs from vertebrate carrion decomposition with or without microbes play a greater role in attracting *Cochliomyia macellaria* to decomposing vertebrate carrion.

In review, detecting and locating carrion is essential not only for carrion-feeding blow flies, but also for nutrient recycling in the community. Blow flies have evolved sensory capabilities, including sensitive visual and olfaction systems to detect VOCs available as public information from suitable resources for oviposition or adult protein meal, such as fresh remains. Carrion olfactory cues can be applied in areas like forensic entomology where insect attraction to and colonization of remains can be used to calculate a time of colonization, which can be related to the time and/or place of death. Once a fly is able to detect the carrion resource, it enters the detection phase of the decomposition process for vertebrate remains (Tomberlin et al. 2011). However, there is still one phase prior to this, known as the exposure phase which encompasses the time from death to

when an insect detects the resource that is available (Tomberlin et al. 2011). Little is currently known about the duration of the exposure phase and the uniformity across species due to inconsistencies between vertebrate models used, storage method and time, as well as the time of day the study is conducted (Tomberlin et al. 2012). Increased knowledge about the exposure and detection phases could make a forensic entomologist's time of colonization estimates a more accurate representation of a true post-mortem interval.

Research Objectives

The research presented was conducted to gain an understanding of how the presence of microbes on vertebrate carrion impacts attraction and colonization of a primary colonizer, *C. macellaria*. I quantified *C. macellaria* attraction to and colonization of mouse carrion with (xenic) and without (axenic) microbes present and characterized the olfactory cues (VOCs) associated with these carrion resources. VOCs are the primary long-distance cue used by primary colonizers to detect and locate a carrion resource. To understand microbe-insect interactions related to decomposition ecology, it is important to investigate multiple steps of the period of insect activity surrounding vertebrate remains from the exposure phase through the acceptance phase. Therefore, the research presented encompasses five objectives in two empirical chapters as described below.

The second chapter will examine objective 1, which will determine the optimum parameters to use when studying *Cochliomyia macellaria* adults in dual-choice cube olfactometer assays. This experiment will elaborate on the existing research conducted using the dual-choice cube olfactometer to assess blow fly adult behavior. *Cochliomyia*

macellaria has yet to be studied in this assay, therefore no baseline information was available to establish effective parameters for current and future research using this species. Results from this study will inform the parameters to use in Objectives 2 and 3 and inform future researchers on how to test for assay optimization.

Objective 1 – Determine the optimal parameters for a dual-choice cube olfactometer assay when testing *Cochliomyia macellaria* adult behavior.

Hypotheses:

H_0: Parameters tested will not provide optimum use of the dual-choice cube olfactometer.

H_a: Parameters tested will provide optimum use of the dual-choice cube olfactometer.

The third chapter has four objectives (objectives 2, 3, 4, and 5) and will focus on how the presence and absence of microbes affect various aspects of *C. macellaria* behavior and VOC production. Objectives 2 and 3 will evaluate the effect of the innate carrion microbiome (Objective 2) and the fly sex and level of female ovarian development (Objective 3) on adult *Cochliomyia macellaria* attraction using a dual-choice cube olfactometer. Previous research has revealed that sex is an important factor influencing insect behavior. Data from the present study will be invaluable as it is the first of its kind to exclude microbes within a carrion resource to determine their effect on primary colonizer behavior and evaluate if the effect is sex specific.

Objective 2 – Determine if the presence of a microbiome on mouse carrion affects adult *Cochliomyia macellaria* attraction over an 8-day decomposition period.

Hypotheses:

H_0: Presence of a microbiome will not affect adult *Cochliomyia macellaria* attraction over time.

H_a: Presence of a microbiome will affect adult *Cochliomyia macellaria* attraction over time.

Objective 3 – Determine the effect of fly sex and female ovarian development on attraction of *Cochliomyia macellaria* to axenic (without microbes) and xenic (with microbes) mouse carrion over an 8-day decomposition period.

Hypotheses:

H_0: Sex and ovarian development does not affect attraction of *Cochliomyia macellaria* adults to axenic and xenic mouse carrion.

H_a: Sex and ovarian development influences attraction of *Cochliomyia macellaria* adults to axenic and xenic mouse carrion.

Objective 4 will evaluate the effect that a microbiome has on the volatile organic profile of a carrion resource and how the profiles change over time. This objective will elaborate on the effects found in objective 2 by determining the compounds being produced by the treatments and exposed to the adults. These data build a bridge between the treatment choices (xenic and axenic mouse carrion) and the behavior choices found in Objectives 2 and 3.

Objective 4 – Determine the change in volatile organic compound profiles of axenic and xenic mouse carrion over an 8-day decomposition period.

Hypotheses:

H_0: Volatile organic profiles of axenic and xenic mouse carrion are not different from one another or over time.

H_a: Volatile organic profiles of axenic and xenic mouse carrion are different from one another or over time.

Objective 5 will evaluate if and how the innate carrion microbiome affects the oviposition behavior of gravid female *Cochliomyia macellaria.* This objective aims to determine the biological impact of oviposition site preference and how microbes can be the driver of oviposition preference. These data can provide valuable insight to the field of decomposition ecology by determining a driver of insect colonization.

Objective 5 – Determine if carrion microbe presence affects oviposition of *Cochliomyia macellaria* on axenic and xenic mouse carrion.

Hypotheses:

H_0: The presence of microbes does not alter oviposition of *Cochliomyia macellaria* on mouse carrion.

H_a: The presence of microbes alters oviposition of *Cochliomyia macellaria* on mouse carrion.

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2. OPTIMIZING A DUAL-CHOICE CUBE OLFACTOMETER DESIGN FOR MEASURING ADULT SECONDARY SCREWWORM (DIPTERA: CALLIPHORIDAE) ATTRACTION TO A RESOURCE*

Overview

Detecting and locating a carrion resource is critical for the reproduction of necrophagous insects and initiating forensically important timelines. Blow flies (Diptera: Calliphoridae) primarily use olfactory cues in the form of volatile organic compounds to locate a suitable resource. Factors governing detecting and locating a resource have been studied using various behavior assays with modifications to suit the experiment design, such as the dual-choice cube olfactometer, which was examined in the current study. Systems optimization ensures biologically relevant and consistent results across replicates. In this study, two responses were measured: 1) leaving cube for either control or treatment and 2) choice between control and treatment. Phenotype (e.g., male, nongravid, gravid) and total blow fly, *Cochliomyia macellaria* (Fabricius) (Diptera: Calliphoridae), number to respond were measured. Four parameters were assessed for their impact on response: 1) adjustment time in cube before trial, 2) trial length, 3) sugar/water presence, and 4) screening type in arms. Approximately, 70% of all

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phenotypes responded to liver with the 30-min adjustment period where only 50% responded with other adjustment periods. Trial length had a significant impact on response (35% increase in the 8 h trial compared to shorter durations); however, significant response to treatment was lost by increasing trial length. The presence of sugar/water decreased gravid and non-gravid response by 35% but did not impact males. Screening had no influence on overall or treatment response. Data indicate experiment design impacts fly response. Future studies should optimize parameters for their given fly population prior to initiating experiments.

Introduction

Carrion provides numerous resources to blow flies (Diptera: Calliphoridae) and other insects such as butterflies (Payne and King 1969), ants (Stoker et al. 1995), and beetles (Rozen et al. 2008). Flesh and associated fluids have a nutritive value for adult and immature blow flies ranging from a protein meal to stimulate oogenesis in adult females to a complete food source for developing larvae (Carter et al. 2007). Blow fly mating is also associated with carrion (Payne 1965). Given its rapid decomposition in temperate and tropic regions (e.g., swine remains skeletonize in 8 d (Payne 1965)), locating it quickly is critical to reduce interspecific and interkingdom competition (Greenberg 1991, Tomberlin et al. 2011).

Blow flies use a variety of cues to locate and assess carrion including visual (Brodie et al. 2014), tactile (Easton and Feir 1991), gustatory (Dethier 1955), and olfactory cues (Paczkowski et al. 2012); however, olfaction appears to be the primary mechanism (Wall and Fisher 2001, Benbow et al. 2015). Olfactory cues, in the form of volatile organic

compounds (VOCs), provide valuable information as related to resource quality (Yan et al. 2018), availability (Vass et al. 2002), and potentially the presence of predators (Flores et al. 2017). The information perceived can be influenced by the carrion species (Paczkowski and Schutz 2011, Cablk et al. 2012, Matuszewski et al. 2019), duration of decomposition (Metcalf et al. 2013) and sex and physiological status of the fly (Liu et al. 2016).

As previously mentioned, VOCs serve as a mechanism regulating blow fly attraction and colonization of carrion. Concentration and class are critical aspects of the VOC profiles regulating these processes. Certain VOCs can be detected at low levels over great distances. For example, concentrations as low as 0.2 M can be detected by *Chrysomya rufifacies* (Macquart) (Diptera: Calliphoridae) of methyl butanoate and methyltrisulfanylmethane (dimethyl trisulfide, DMTS) using a GC-EAD (Kolodij 2015). The primary screwworm *Cochliomyia hominivorax* (Coquerel) (Diptera: Calliphoridae) is capable of travelling more than three kilometers to find a resource, and even up to 25 km when in unfavorable habitats with scarce resources (Mayer and Atzeni 1993).

Determining when blow flies arrive and colonize human or other vertebrate remains is a critical aspect of forensic entomology. By determining the time of colonization, a minimum postmortem interval can be estimated, given certain assumptions are met (Catts and Haskell 1990, Tomberlin et al. 2011) . Such information is valuable for determining events leading to the demise of the individual or duration of neglect and abuse (Benecke et al. 2004); however, it should be noted that arrival and colonization can vary depending on the cues detected by the insects. Determining which cues regulate such

behavior is critical for increasing precision and accuracy when estimating a postmortem interval from a time of colonization estimate (Matuszewski 2011, Tomberlin et al. 2011).

In the southern United States, *Cochliomyia macellaria* (Fabricius) (Diptera: Calliphoridae), known as the secondary screwworm, is commonly found throughout the warmer months (Hall 1948). In Texas where this study was conducted, *C. macellaria* is most commonly active from late spring into late fall and is usually a primary colonizer of carrion (Tenorio et al. 2003). The close association of blow flies, like *C. macellaria*, with decomposing remains has resulted in their recognition as a key species used to estimate time of colonization in forensic entomology investigations (Boatright and Tomberlin 2010, Sanford 2017).

Different devices have been created to observe and explore the behavior of insects. These devices have been modified and used to assess the behavior of necrophilous insects in the presence of cadaveric VOCs. Designs for such equipment have diversified to encompass a number of factors associated with such research (e.g., number of treatments, size of the insect, locomotion of insect). Dipteran behavior has been assessed using a Y-tube olfactometer (Urech et al. 1994, Erler et al. 2006, Frederickx et al. 2012, Brundage et al. 2017), a four-arm olfactometer (Yu et al. 2013), and a dual-choice cube olfactometer (Tomberlin et al. 2012) to name a few.

The dual-choice cube olfactometer is a promising design as a behavior assay, but there are limited data on its use. In order to enhance research with this design, parameters need to be optimized to ensure biologically relevant results and consistency across replicates. In this study, we determined the impact of the following factors on adult *C.*

macellaria response; 1) adjustment time, 2) trial length, 3) sugar and water status, and 4) insect screening type to identify the optimal parameter for the dual-choice cube olfactometer.

Materials and Methods

Colony Maintenance

Flies used for this research were from a colony maintained at the Forensic Laboratory for Investigative Entomological Sciences (F.L.I.E.S. Facility) at Texas A&M University (College Station, TX). *Cochliomyia macellaria* adults were housed in 30 × 30 × 30 cm BioQuip bug dorms (BioQuip Inc., CA) located in a rearing room with environmental conditions maintained at 23.8°C, and a 14:10 h L:D cycle. Adult flies were provided with a 3:1 sugar: powdered milk mixture and water immediately after emergence and replenished ad libitum. Adults at approximately 2-d-old were provided with approximately 10 ml of bovine blood (Rosenthal Meat Science and Technology Center, Texas A&M University) in a petri dish every other day for 6 d to stimulate oogenesis. Adults were then provided access to 10 ml of bovine blood along with 10 g of bovine liver (Rosenthal Meat Science and Technology Center, Texas A&M University) in a petri dish. After 48 h, adults were provided with approximately 50 g of bovine liver as a substrate for oviposition. Adults had access to the bovine liver for 24 h. At the end of this period, the liver was replaced with new liver for adults to lay subsequent egg clutches. Liver and eggs were transferred into a 0.94-liter wide mouth mason jar (Ball, Broomfield, CO) filled approximately half full of vermiculite (Producer's Cooperative Association, Bryan, TX) for larval development. Larvae were provided additional bovine liver which was

replenished ad libitum. The adult flies used in these trials were 7–9 d old. All forms and paperwork required to obtain blood and tissue from harvest for use in nonhuman consumptive experiments were approved and maintained on file.

Cube Design

The dual-choice cube olfactometer ('cube' as depicted in Fig. 2.1) was constructed out of 0.56 cm clear plexiglass (Lowe's, Mooresville, NC) cut into 45 × 45 cm squares. Two sides had a 11.5-cm circle cut in the center and served as exit sites for the arms of the olfactometer. The cubes were assembled using Plastic Weld (Plastruct, City of Industry, CA) with the two holes on opposing sides to allow connection of PVC arms. The PVC arms consisted of a 10-cm diameter PVC pipe 30.5 cm in length (The Home Depot Inc., Atlanta, GA). The side inserted into the dual-choice cube olfactometer had a 7.6 cm to 3.8 cm reducing coupling (The Home Depot Inc.). The distal end of the tube was covered with Phifer charcoal fiberglass insect screening (The Home Depot Inc.) held in place by a 11.5-cm 90-degree long-turn elbow (The Home Depot Inc.). Two non-scented Trapper Max glue traps (Bell Laboratories, Madison, WI) were placed inside of the PVC pipe between the reducing coupling and the insect netting to trap any flies that entered that arm. The distal side of the elbow housed the top of a 0.94-liter wide mouth mason jar (Ball, Broomfield, CO), containing the treatment designated for that arm.

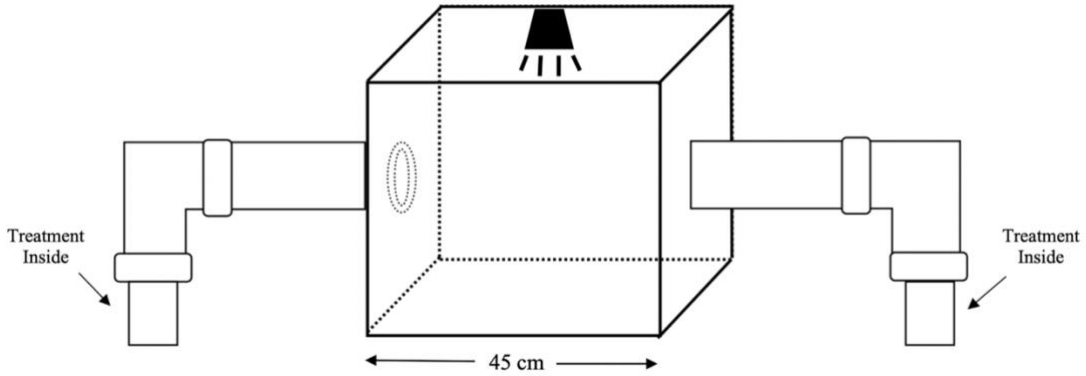

Figure 2.1 Diagram of the dual-choice cube olfactometer.
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Each individual cube required its own light source. Commercial Electric 23 cm LED Under Cabinet Lights in Soft White were used to supply light to the individual cubes. Each cube was covered in a black cloth sleeve made of 90% Nylon, 10% Spandex fabric (Jo-Ann Stores, Inc., Hudson, OH) to reduce interactions between olfactometers and external light sources. Sleeves were modified to allow the arms of the olfactometer to be connected as well as positioning of the light source. Sleeves were washed with all Free Clear liquid laundry detergent (Henkel Consumer Goods, New Province, NJ) before each use and allowed to air dry. Cubes were washed with a 1% Alconox Powdered Precision Cleaner solution according to the manufacturer's instructions (Alconox, Inc., White Plains, NY) followed by 70% ethanol and allowed to air dry before each use. The PVC arms were washed with a 1% Alconox Powdered Precision Cleaner solution according to manufacturer's instructions (Alconox, Inc.), 70% ethanol, and acetone, and allowed to air dry before each use. Flies were transferred from their rearing cage to the cube via hand

vacuum before each trial. The arms were taken off of the cubes and replaced with 10.2 cm PVC caps (The Home Depot Inc.) at the conclusion of each trial.

General Experiment Design

The following experiment setup was used to test each of the factors listed below independently. Approximately 200 flies at equal sex ratio were released into the center of each cube at approximately 0800 h. Food and water were not provided. Olfactometer treatments were either an empty mason jar or one containing 35 g of freshly thawed bovine liver. Treatment location (i.e., arm of olfactometer) was randomly assigned. Once the experiments concluded, sticky traps were removed from the arms, and the cube containing remaining flies was placed in a −20°C freezer. After 48 h, flies were removed from the freezer, sexed via interocular distance, and female ovarian development determined by dissection of the abdomen or presence of eggs laid on the sticky trap. Flies collected on sticky traps were also tabulated in the same categories. Three trials were conducted for each factor tested with one replicate each.

Adjustment Time

The amount of time allowed for flies to explore prior to initiating an experiment could impact fly response. Five cubes were set up as previously described sans sugar and water. Adjustment times varied from 0, 15, 30, 45, to 60 min. Once the adjustment period had expired, the attractants and controls were attached to the olfactometers and experiment initiated.

Trial Length

Trial length was examined to determine the optimum amount of time to allow for the maximum response while remaining biologically relevant. Longer trial lengths should result in a higher response rate but may not be biologically relevant or reliable. Using experiment design previously described, fly response to treatments over time (2, 4, 6, and 8 h) was recorded.

Sugar and Water Status

Sugar and water presence in the cubes could act as a response deterrent or distraction. Six cubes were set up as previously described. Three of the cubes contained 2 g of sugar in one petri dish and 10 ml of water in another petri dish centered in the cube. The other three cubes did not contain sugar or water. This parameter will hereafter be referred to as 'status'.

Mesh Type

Insect netting that is re-used could cause a response bias due to exposure to prior flies and VOCs emitted from the treatments, inducing a greater response. Arms of three cubes had new insect screening and the other cubes contained screening that had been used on a liver treatment side in a prior trial. This parameter will hereafter be referred to as 'type'.

Statistical Analysis

Data were analyzed with RStudio version 1.2.1335 (R Core Team 2013). The assumptions of normality were tested using a Shapiro– Wilk test, and equal variances were tested using a Bartlett's test. All assumptions of an analysis of variance (ANOVA) were

met unless otherwise stated. Alpha was set at 0.05, and nonsignificant interactions were removed from the analysis. Tukey's HSD test was conducted after a significant ANOVA result.

Results

Adjustment Time

Pooled data across sex and physiological state (i.e., male, gravid, and non-gravid combined) for fly response were not significant ($F_{4,10} = 1.468$, $P = 0.283$) for adjustment time and providing a response (i.e., departing center cube and choosing liver or control). On average, 57% responded at 0 min and the highest response was at 45 min (66%). When analyzed by sex and physiological state, adjustment time ($F_{4,16} = 7.458$, $P = 0.001$), sex ($F_{2,16} = 12.809$, $P < 0.001$), trial ($F_{2,16} = 13.776$, $P < 0.001$), trial × sex ($F_{4,16} = 4.314$, $P = 0.014$), and adjustment time × trial ($F_{8,16} = 4.663$, $P = 0.004$) all had a significant impact on response percentage while adjustment time × sex ($F_{8,16} = 0.439$, $P = 0.880$) was not significant. Collectively, gravid females responded 13% ($P < 0.05$) more than males and non-gravid females (Fig. 2.2A). For those responding to the treatment (i.e., liver) or control, pooled fly response was not significant ($F_{4,8} = 2.194$, $P = 0.159$) for adjustment time but was for trial ($F_{2,8} = 7.862$, $P = 0.012$). No interactions were tested due to a lack of available degrees of freedom in the analysis.

When analyzed by sex and physiological state, adjustment time ($F_{4,16} = 12.003$, $P < 0.001$), trial ($F_{2,16} = 44.947$, $P < 0.001$), and adjustment time × trial ($F_{8,16} = 5.764$, $P = 0.001$) all had a significant impact on level of response to liver versus control, while sex ($F_{2,16} = 0.618$, $P = 0.551$), trial × sex ($F_{4,16} = 1.256$, $P = 0.327$) adjustment time × sex ($F_{8,}$

$_{16}$ = 0.545, P = 0.805) did not. Gravid females with 0 min ($F_{1, 4}$ = 7.962, P = 0.047), gravid females with 30 min ($F_{1,4}$ = 35.41, P = 0.004), males with 30 min ($F_{1, 4}$ = 17.83, P = 0.013), and non-gravid females with 30 min ($F_{1, 4}$ = 9.576, P = 0.036) responded significantly more to liver rather than the control (approximately 25, 45, 40, and 35%, respectively; Fig. 2.2B). Although not statistically significant ($F_{1, 4}$ = 6.624, P = 0.061), non-gravid females at 0 min trended toward responding more to liver (approximately 20% greater).

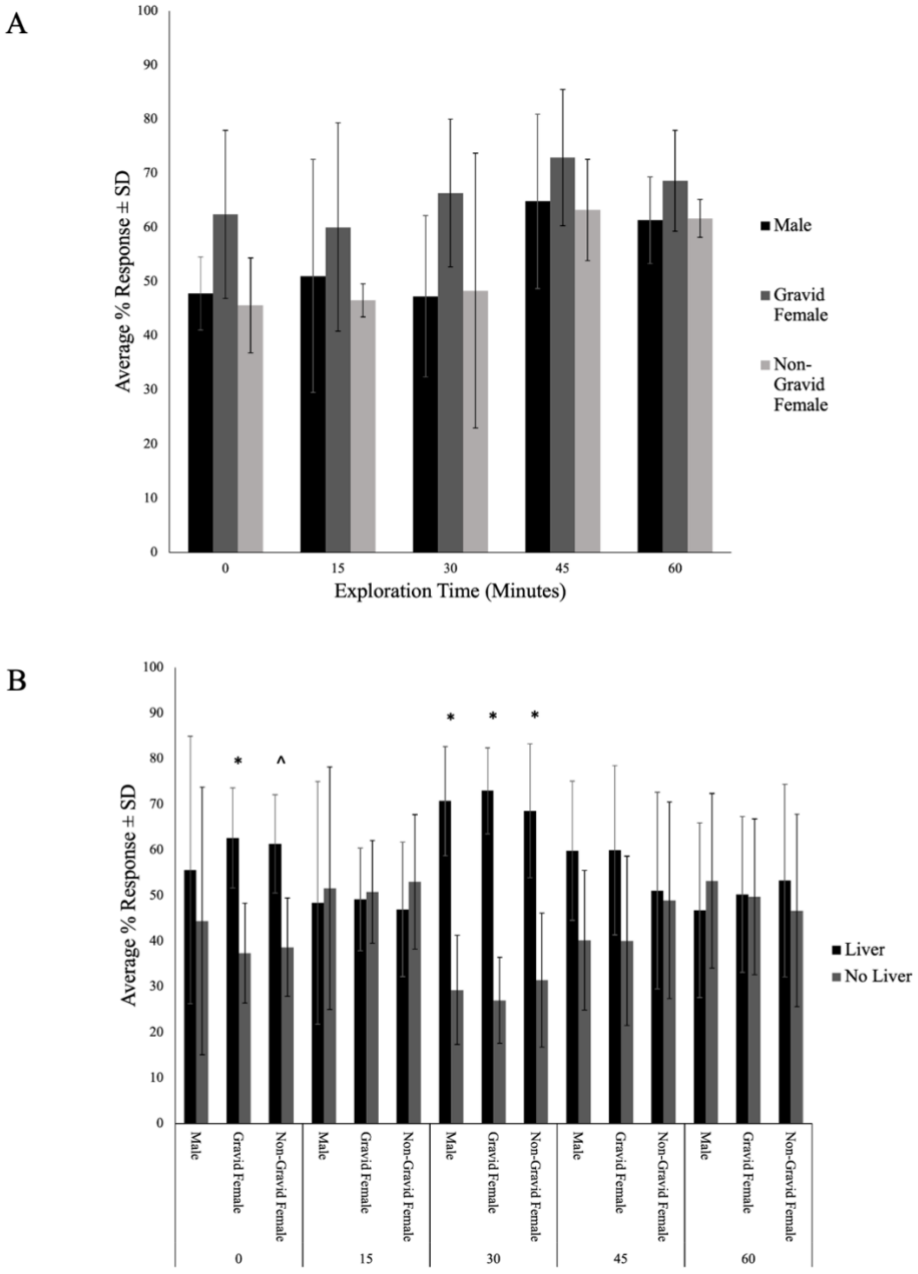

Figure 2.2 Average percent ± SD response of approximately 200 7-9-d-old male, gravid, and non-gravid C. macellaria in a dual-choice cube olfactometer maintained at approximately 21°C to treatments with a 0, 15, 30, 45, or 60 min adjustment period for (A) overall response by phenotype and (B) response to liver and control treatments by phenotype; [1]* significance of $P \leq 0.05$; [2]^ significance of $P \leq 0.10$.
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Trial Length

Pooled data across sex and physiological state for fly response (i.e., departing center cube and choosing liver or control) were significant ($F_{3, 8} = 10.11$, $P = 0.004$) for trial length. Flies responded significantly more in the 8-h trial (approximately 65%) when compared with the 2 (approximately 26%), 4 (approximately 34%), and 6-h (35%) trials.

When analyzed by sex and physiological state, trial length ($F_{3, 12} = 28.708$, $P < 0.001$), sex ($F_{2, 12} = 4.638$, $P = 0.032$), trial length × sex (Fig. 2.3A; $F_{6, 12} = 3.875$, $P = 0.021$), and trial length × trial ($F_{6, 12} = 4.196$, $P = 0.016$) had a significant impact on level of response, while trial ($F_{2, 12} = 2.055$, $P = 0.170$) and sex × trial ($F_{4, 12} = 1.880$, $P = 0.178$) did not. Males had the greatest percent response in the 2-h trial (approximately 10% greater); however, females had the greatest response for other trial lengths (approximately 5–30% greater).

For those responding to the treatment (i.e., liver) or control, pooled fly responses were not significantly different ($F_{3, 6} = 0.851$, $P = 0.515$) based on trial length, although trial was ($F_{2, 6} = 5.333$, $P = 0.046$). No interactions were tested due to a lack of available degrees of freedom in the analysis. Flies were equally attracted to the treatment and control across statuses when pooling sex and physiological state in the analysis.

When response was analyzed by sex and physiological state, interactions were not significant ($P > 0.05$). Subsequent analysis determined trial ($F_{2, 28} = 5.712$, $P = 0.008$) significantly impacted the level of response to the liver treatment while sex ($F_{2, 28} = 0.898$, $P = 0.418$) and trial length ($F_{3, 28} = 2.528$, $P = 0.077$) did not. Gravid females at 2 h ($F_{1, 4} = 7.934$, $P = 0.048$) and males at 2 h ($F_{1, 4} = 11.19$, $P = 0.028$) responded significantly more

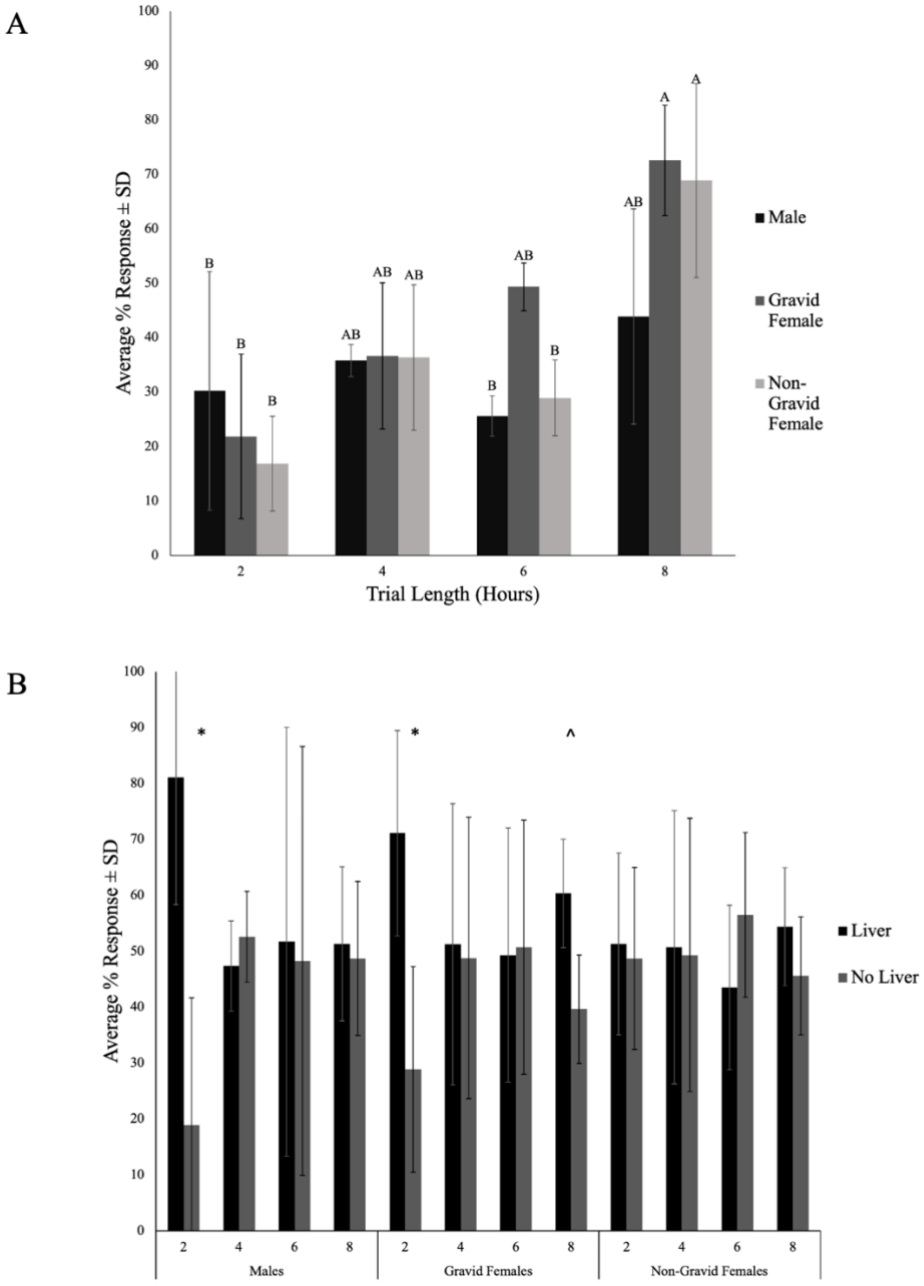

Figure 2.3 Average percent ± SD response of approximately 200 7-9-d-old male, gravid, and non-gravid C. macellaria in a dual-choice cube olfactometer maintained at approximately 21°C to treatments for 2, 4, 6, or 8 h for (A) overall response by phenotype and (B) response to liver and control treatments by phenotype; [1]* significance of $P \leq 0.05$; [2]^ significance of $P \leq 0.10$.
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to liver rather than control (Fig. 2.3B). Although not statistically significant ($F_{1, 4} = 6.832$, $P = 0.059$), gravid females during the 8-h experiment show a trend toward responding to the liver.

Sugar and Water Status

Pooled data across sex and physiological state for fly response were significant ($F_{1, 4} = 9.02$, $P = 0.039$) for sugar and water status and providing a response (i.e., departing center cube and choosing liver or control). No interactions were tested due to a lack of available degrees of freedom in the analysis. Overall, 63% of flies remained in the cube when sugar and water were provided compared to 38% when they were absent (Fig. 2.4A).

When analyzed by sex and physiological state, sex ($F_{2, 4} = 0.014$, $P = 0.071$), trial ($F_{2, 4} = 0.00419$, $P = 0.302$), and sex × trial ($F_{2, 4} = 0.13651$, $P = 0.064$) did not significantly influence the level of response while status ($F_{1, 4} = 159.294$, $P < 0.001$), status × trial ($F_{2,4} = 53.369$, $P = 0.001$), and status × sex ($F_{2, 4} = 68.573$, $P < 0.001$) did. Sugar and water presence significantly decreased gravid and non-gravid female response from approximately 70% to approximately 35% but had no effect on males (Fig. 2.4B).

For those responding to the treatment (i.e., liver) or control, pooled fly responses were not significantly different based on status ($F_{1, 2} = 0.316$, $P = 0.631$) or trial ($F_{1, 2} = 0.518$, $P = 0.659$). No interactions were tested due to a lack of available degrees of freedom in the analysis. Flies were equally attracted to the treatment and control across statuses when pooling sex and physiological state in this analysis.

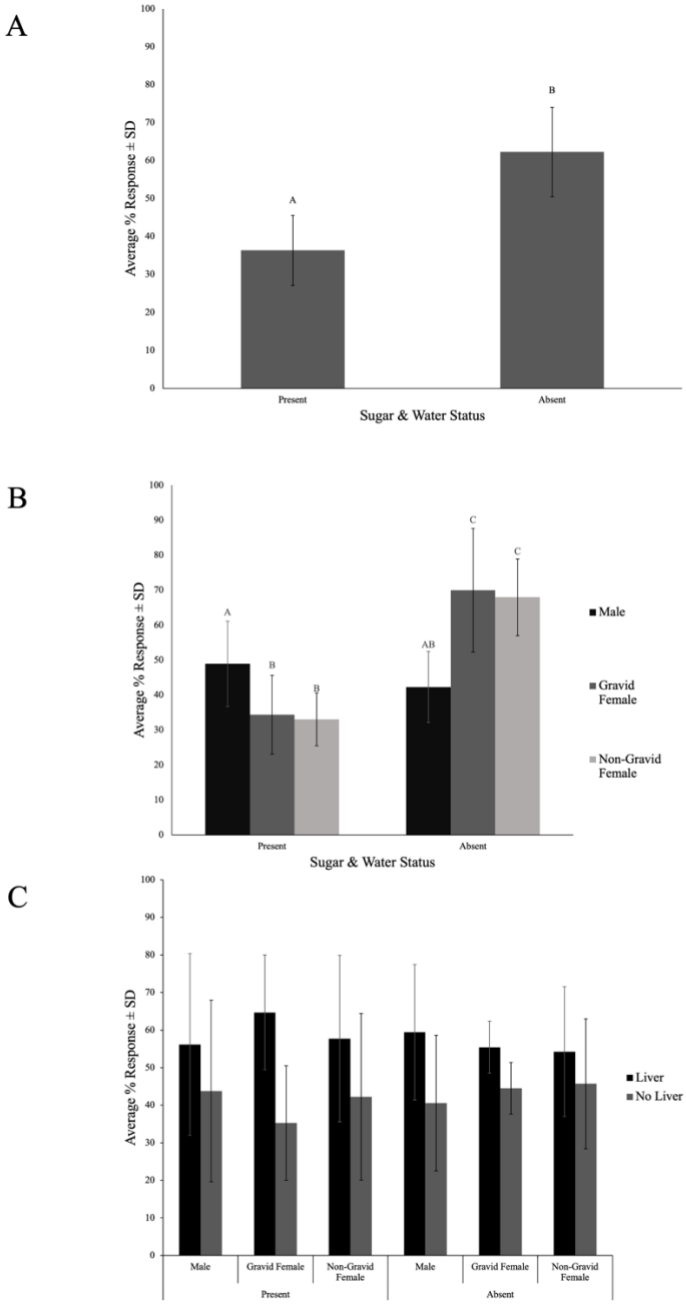

Figure 2.4 Average percent ± SD response of approximately 200 7-9-d-old male, gravid, and non-gravid C. macellaria in the presence and absence of sugar and water in a dual-choice cube olfactometer maintained at approximately 21°C for 8 h for (A) overall response with pooled phenotype, (B) overall response by phenotype, and (C) response to liver and control treatments by phenotype.
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When response was analyzed by sex and physiological state, interactions were not significant ($P > 0.05$). Subsequent analysis determined sex ($F_{2, 12} = 0.104$, $P = 0.902$), status ($F_{1, 12} = 0.187$, $P = 0.673$), and trial ($F_{2, 12} = 2.463$, $P = 0.127$) did not influence response to liver (Fig. 2.4C). Across sexes and treatments, there was a nonsignificant trend toward response to liver (approximately 9–30% higher) when compared with the control (Fig. 2.4C).

Mesh Type

Pooled data across sex and physiological state for fly response were not significant ($F_{1, 2} = 0.334$, $P = 0.622$) for mesh type (new vs used) and providing a response (i.e., departing center cube and choosing liver or control). Trial was also not significant in this analysis ($F_{1, 2} = 4.647$, $P = 0.177$). No interactions were tested due to a lack of available degrees of freedom in the analysis. Approximately 50–60% of adult flies entered an arm, regardless of the mesh type used (Fig. 5A).

When response was analyzed by sex and physiological state, interactions were not significant ($P > 0.05$). Subsequent analysis determined type ($F_{1, 12} < 0.001$, $P = 0.999$), sex ($F_{2, 12} = 1.928$, $P = 0.188$), trial ($F_{2, 12} = 1.762$, $P = 0.213$) did not significantly impact response. Although sex was not a significant factor, non-gravid females tended to respond approximately 10 and 15% less than gravid females and males respectively (Fig. 5B).

Regarding the response to treatment (i.e., liver) or control, percentage of pooled adult response to liver versus control was not significantly different based on mesh type ($F_{1, 2} = 0.866$, $P = 0.450$) or trial ($F_{2, 2} = 4.443$, $P = 0.184$). No interactions were tested

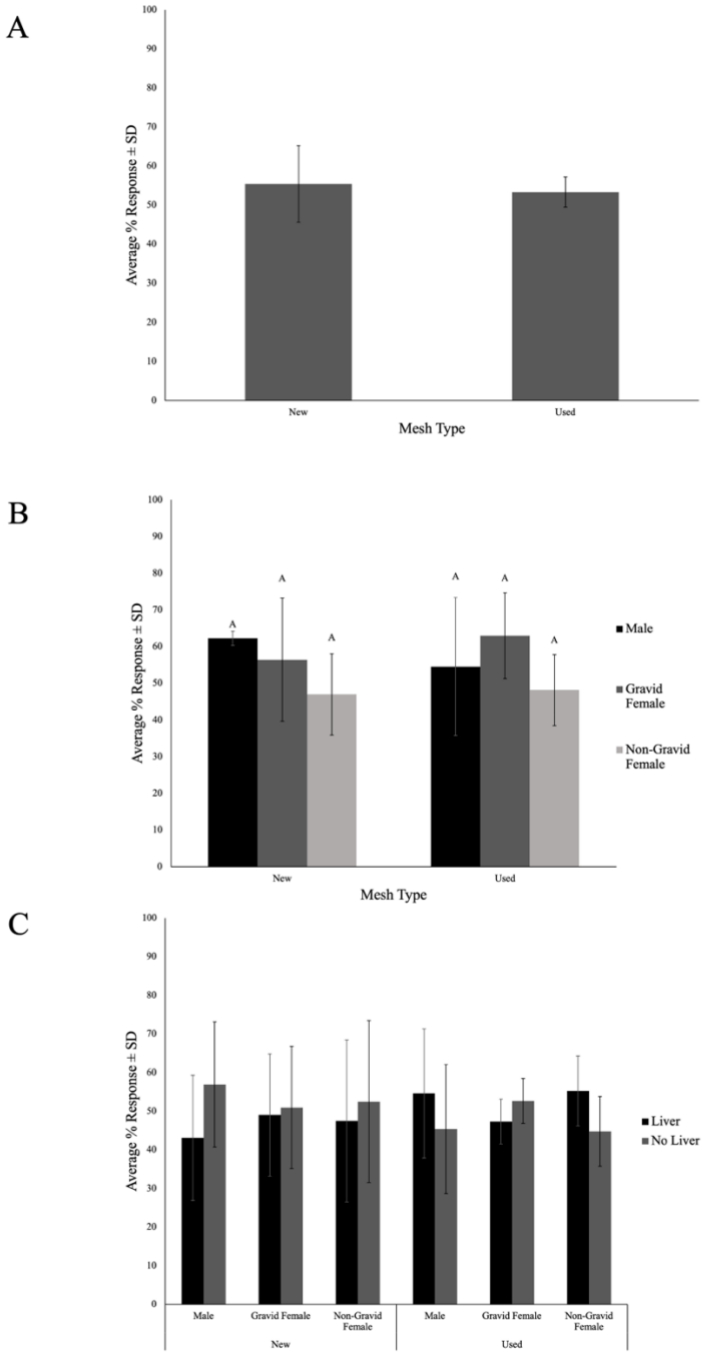

Figure 2.5 Average percent ± SD of response of approximately 200 7-9-d-old male, gravid, and non-gravid C. macellaria in a dual-choice cube olfactometer maintained at approximately 21°C for 8 h for (A) overall pooled sex response, (B) overall response by phenotype.
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due to a lack of available degrees of freedom in the analysis. Flies were equally attracted to the treatment and control across statuses when pooling sex and physiological state in the analysis. When response was analyzed by sex and physiological state, interactions were not significant ($P > 0.05$). Subsequent analysis determined trial ($F_{2, 12} = 11.636$, $P = 0.001$) was a significant factor, but sex ($F_{2, 12} = 0.211$, $P = 0.812$) and type ($F_{1, 12} = 1.912$, $P = 0.191$) did not influence response to the liver treatment when compared with the control (Fig. 5C).

Discussion

The results of this study indicate that specific parameters associated with examining blow fly response to treatments with a dual-choice cube olfactometer should be evaluated prior to conducting experiments including acclimation time, trial length, sugar and water status, and mesh type. Establishing these parameters before initiating an experiment will ensure the most accurate results regarding attraction to a resource are obtained using the dual-choice cube olfactometer. Once optimization of a behavior assay is determined, the true effects of the treatments can be explored rather than effects from variation in any of the parameters. Furthermore, utilizing defined categories as related to sex and physiological state is crucial for determining specific blow fly responses. Utilizing pooled data (i.e., combining sex and physiological state) provided to be a coarse investigation and in this case, not informative (i.e., response to variables tested masked). With regards to the parameters measured, adjustment period did not have an effect on level of response when looking at pooled sex or by sex analyses; however, allowing the flies 30 min to settle in the olfactometer prior to initiating the experiment resulted in 40% greater

difference in terms of response to treatment versus control for each sex (Fig. 2B). Although the level of response increased from 2 h to 8 h trial lengths when looking at pooled data, the treatment effect diminished after 2 h and therefore, trial length for *C. macellaria* should be restricted to 2 h. In general, providing sugar and water to the flies in the central cube suppresses responses to the control and treatment by 25% and therefore should not be used. Of course, this was phenotype specific with males increasing response by approximately 5% and females decreasing response by approximately 35%. Mesh type did not affect response percentage overall or by sex.

Placing adult insects in a new environment using an insect vacuum without environmental adjustment did not impact their overall response but influenced their treatment response. The 30 min period appeared to be sufficient for the adults to habituate to their new environment, as this was the only treatment time that resulted in a significantly higher response to the treatment compared to the control for all three phenotypes (Fig. 2B). This length of time has been used in a previous study using *C. macellaria* which found significant results in response to VOC treatments on liver for all three phenotypes (Chaudhury et al. 2017). For wingless *Phormia regina*, (Meigen) (Diptera: Calliphoridae) adults, there was a significant decrease in repetitive hopping as a mechanism to begin flight in just 10 min indicating habituation (Dethier 1993).

Trial length influenced overall response and treatment effect for *C. macellaria*, as a longer trial length allowed for greater response; however, the treatment effect diminished after only 2 h (Figs 4 and 5). Treatment effects were significant in prior studies using a 1-h and 3-h trial length (Chaudhury et al. 2017), which aligns with results in this study

(highest at 2 h and diminishes thereafter). This could be due to saturation of the VOCs within the cube or habituation to the odors present, although further research would have to be conducted to determine the saturation point. A number of studies examine habituation in respect to odor or compound stimulus, showing a decreased aversion to the stimulus compound such as adult *Drosophila* (Diptera: Drosophilidae) with previous exposure to peppermint oil, 7% ethanol medium, and piperidine (Jaenike 1982). Regarding the blow fly *Cochliomyia hominivorax* (Coquerel) (Diptera: Calliphoridae), habituation (decreased attraction) to decomposition odors was shown to occur between 15 and 30 min after initial exposure to treatments (Fletcher and Turner 1973, Adams et al. 1979). Using a Y-tube olfactometer determined a trial length of 2-min was not statistically different than a 5-min trial for *C. macellaria* (Brundage et al. 2017); however, this could not be determined using a dual-choice cube olfactometer due to the differences in design.

The species tested can potentially impact the trial length needed for sufficient response. Life-history traits are specific for each blow fly species and can impact the parameters used in experiments. *Cochliomyia macellaria* provided significant results in the 2-h trial while failing to do so in longer trials. *Lucilia sericata* (Meigen) (Diptera: Calliphoridae) was given 24 h to respond in a prior study using the dual-choice cube olfactometer and provided significant results, revealing the impact of species on response time (Ma et al. 2012, Tomberlin et al. 2012). This difference could be due to the differences in life-history traits between the two species. *Cochliomyia macellaria* tends to prefer warmer weather and is commonly found in the southeastern United States in the spring and summer months (Hall 1948, Tenorio et al. 2003), where *L. sericata* tends to

prefer cooler weather (Hall 1948). The temporal difference in activity could also be related to rate of decomposition differences in the summer months (up to 315 degree hours for the fresh stage) compared to winter months (up to 3,185 degree hours for the fresh stage) (Benbow et al. 2013). Temporal behavior differences are also seen in *Culex tarsalis* (Coquillett) (Diptera: Culicidae) with regard to host preference (Tempelis and Washino 1967), *Musca autumnalis* (DeGeer) (Diptera: Muscidae) with regard to activity (Peterson and Meyer 1982), and robber flies of various species (Diptera: Asilidae) with regard to activity (Mccravy and Baxa 2011). Trail length should be investigated when using a new species and/or a new behavior assay to determine the optimal duration.

Sugar and water served as a distraction in the olfactometer for gravid and non-gravid females. Dipterans are capable of detecting moisture in the surrounding environment (Enjin et al. 2016), which could indicate that flies responding in the 'sugar and water absent' treatment were responding to the liver treatment as a source of hydration rather than protein or an oviposition substrate. *Drosophila melanogaster* (Meigen) (Diptera: Drosophilidae) is capable of detecting and differentiating sugars and amino acids with olfaction and will locate a resource depending on their deficiency (Lin et al. 2019), further enforcing the idea that when sugar and water are present, adults would leave the olfactometer to search for the protein resource.

Age of adults used in the behavior assay could have influenced the response and therefore should be based of the population used in the experiment. In the colony used for this study, 7–9 d is the optimal age for reproduction and oviposition because this is the age where females are beginning to lay eggs. Previous studies have used flies older than

7–9 d old (Chaudhury et al. 2017) while other studies conducted in the same facility as this study have also used 7-d-old *C. macellaria* adults (Brundage et al. 2017). A previous study using the dual-choice cube olfactometer demonstrated that the protein source provided to adults has a greater impact on level of response when compared with age of the adults used (Tomberlin et al. 2012).

A possible limitation of using a dual-choice cube olfactometer is the number of resources used in the distal end of the arms when testing the parameters. Bovine liver was used in one arm of the olfactometer while the other arm was an empty control in order to examine the parameters recommended for the dual-choice cube olfactometer (adjustment time, trial length, sugar and water status, and mesh type). This colony is reared on bovine liver and could have a predisposition in the response preference, with respect to the chemical legacy hypothesis (Corbet 1985). Due to the treatments being cube parameters and not arm choices, this limitation does not significantly affect this study, but could affect experiments in the future if examining choices using a 'familiar' substance as a control.

This study determined that specific parameters should be explored and optimized when assessing Calliphoridae behavior using a dual-choice cube olfactometer. If future studies are conducted with the dual-choice cube olfactometer, individuals should conduct preliminary work to optimize the parameters based on the species used. These data establish a foundation for conducting research addressing the attraction phase of the pre-colonization interval used in time of colonization estimates in forensic entomology investigations (Tomberlin et al. 2011).

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3. VERTEBRATE MICROBIOME SERVES AS A MECHANISM PARTIALLY REGULATING BLOW FLY (DIPTERA: CALLIPHORIDAE) ATTRACTION AND COLONIZATION

Introduction

Decomposition is a universal but complex process with numerous players (i.e., structure) with diverse roles (i.e., function) involved. The fauna efficiently recycle essential nutrients back into their environments, acting as a bridge between the brown and green food webs, and typically specialize in autotrophic or heterotrophic necromass. The process of nutrient recycling has been vastly studied, and a long history of studies have focused on the recycling of vertebrate necromass, known as carrion (Mégnin 1894, Payne 1965, Kuusela and Hanski 1982, Wilson and Wolkovich 2011, Pechal et al. 2013b). Carrion nutrient recycling, the carrion-feeding recyclers, and their mostly predictable pattern of arrival and colonization in have been applied to the legal system to infer forensically relevant timelines including the time of colonization (TOC) and postmortem interval (PMI).

Forensic entomologists have divided the PMI into two phases: the pre-colonization interval which occupies the time from death until insect colonization, and the post-colonization interval which occupies the time between insect colonization and the discovery of remains (Tomberlin et al. 2011). Forensic entomology practitioners can use the age of the larval insects, mostly Diptera, recovered from remains to infer a time of colonization (TOC) estimate and therefore the post-colonization interval. Traditionally the duration of the pre-colonization interval has been neglected, but various studies have

shown factors that can delay insect colonization and increase the pre-colonization interval (Matuszewski 2011, Matuszewski and Szafałowicz 2013, Mohr and Tomberlin 2014, 2015a, Pittner et al. 2020). Although exposure of remains to primary colonizers, such as blow flies (Diptera: Calliphoridae), can be immediate after death of the individual, it takes time for an adult fly to detect and locate the remains before colonization can occur. During this time, the microbial community within the remains is beginning to undergo rapid changes.

Of course, while the individual is alive, the human immune system regulates the microbiome by utilizing physical barriers such as epithelial cells to compartmentalize the intestinal microbiota (Konrad et al. 2006) or chemically such as raising the body temperature to activate genes responsible for producing immune cells for an inflammatory response (Harper et al. 2018). Once the immune system ceases functioning after death, the microbes are no longer regulated by the host and gain access to all the nutrients and tissue available in the human body. This leads to a proliferation in the number of microbes and competition among taxa for available nutrients. In the proximal large intestine of humans, *Bacteroidetes* and *Lactobacillus* can be used as quantitative markers of the PMI duration because their relative abundance declines exponentially as PMI increases in a significant and repeatable manner (Hauther et al. 2015). There is also a distinct shift in community from aerobic to anaerobic bacteria in areas such as the mouth, GI tract, and the general body cavity (Hyde et al. 2013). Tissue type and the detection/sequencing technology used can determine which microorganisms are detected and to what level of reliability they can be used. Although studies with human remains have been limited due to donor availability,

research with human surrogates such as swine and mice are valuable, as they can support well-replicated studies and use of destructive sampling techniques. Such studies have also shown a repeatable and predictable pattern of postmortem changes in microbiome composition, known as the "microbial clock" (Metcalf et al. 2013, Pechal et al. 2013b). Mice incorporated with a human microbiota show an increase of enterobacteria and enterococci by 3 to 5 orders of magnitude in the intestine after death (Heimesaat et al. 2012).

As microbes battle for the newly available resource, they are translocating throughout the body as early as 5 min after death (Heimesaat et al. 2012) while emitting metabolic byproducts and chemical cues in the form of volatile organic compounds (VOCs) (Janzen 1977). Such compounds are emitted throughout the decomposition process, with over more than 475 specific VOCs identified during human burial (Verheggen et al. 2017). Notably, 225 of those compounds are also correlated with swine decomposition (Dekeirsschieter et al. 2012).

Dipterans that utilize carrion as a resource for reproduction and development have evolved the ability to detect and locate this resource extremely efficiently due to the ephemeral nature of the resource. Adult blow flies use their antennae to detect long distance olfactory cues with the ability to detect trace amounts of compounds and travel several kilometers to locate a suitable resource for their offspring. For example, *Chrysomya rufifacies* (Coquerel) (Diptera: Calliphoridae) can detect methyl butanoate (butanoic acid, methyl ester) and methyltrisulfanylmethane (dimethyl trisulfide, DMTS) at concentrations as low as 0.2 M using a CG-EAD system (Kolodij 2015). Another

species, *Cochliomyia hominivorax* (Coquerel) (Diptera: Calliphoridae), has been documented travelling more than three kilometers to locate a suitable resource, and up to 25 kilometers in an unfavorable habitat (Mayer and Atzeni 1993).

Bacteria have a great influence on the VOC profile produced during decomposition and are known to affect blow fly behavior. Microbial VOCs (mVOCs) have been shown to alter attraction of a fly to a resource by potentially acting as a "camouflage" for predators or allowing individuals to identify conspecifics (Brundage et al. 2017). Gravid females use mVOCs to determine if a substrate is suitable for oviposition, and have been suggested as a required prerequisite (DeVaney et al. 1973). Examples of mVOCs that are known adult fly attractants include indole, putrescine, phenol, and lactic acid (Ma et al. 2012) but the level of attraction can be influenced by the sex and physiological state of the fly (Liu et al. 2016a).

Cochliomyia macellaria (Fabricius) (Diptera: Calliphoridae) is a common primary colonizer of carrion in the southern United States (Hall 1948). In Texas, where this study was conducted, *C. macellaria* is especially common and active in the warmer months spanning from the late spring to the early fall and is a primary colonizer of carrion (Tenorio et al. 2003). Due to their close association with remains, *C. macellaria* is known as a key species used to estimate time of colonization in forensic investigations (Boatright and Tomberlin 2010, Sanford 2017).

There is promising information leading to the recognition that microbes have a great role in the interaction between adult Diptera and their colonization of decomposing remains (Tomberlin et al. 2017) but no study has excluded microbes from the carrion

resource to study this impact. In the present study, we examine four different objectives: microbiome impact on attraction, sex and ovarian development impact on attraction, microbiome impact on volatile organic compounds, microbiome impact on oviposition by *C. macellaria*. Such information could prove vital for greater understanding of the pre-CI and its relations to the PMI of decomposing human remains.

Methods

Supply Preparation

All equipment and supplies were sterilized with a Tuttnauer 2340E Autoclave (Tuttnauer, Breda, PR, NL) prior to use. Euthanized 4-6 week old, ~30g male germ free (axenic) and specific pathogen free (xenic, microbiome is proprietary) mice from the C57BL/6J substrain of *Mus musculus* (Rodentia: Muridae) were purchased from the Baylor College of Medicine Gnotobiotics Core (Houston, Texas, USA). Axenic and xenic male mouse carcasses were received the day prior to experiment initiation in individual shipping containers which were kept at 4°C until use in the experiment the following morning. IACUC approval was not needed given the animals were culled from the colony during routine maintenance and not euthanized specifically for this project.

Insect Colony Maintenance

Adult *C. macellaria* used for this research were from a colony maintained at the Forensic Laboratory for Investigative Entomological Sciences (F.L.I.E.S. Facility) at Texas A&M University (College Station, TX, USA). This colony originated in 2014 with wild type individuals added approximately every 10 generations. Flies were housed in 30

x 30 x 30 cm BioQuip bug dorms (BioQuip Inc., CA, USA) immediately after emergence and maintained at 23.8° C with a 14:10 h light: dark cycle while provided with a 3:1 sugar: powdered milk mixture and water; replenished *ad libitum*. Adults at 2-d-old were provided with 10 ml of bovine blood (Rosenthal Meat Science and Technology Center, Texas A&M University, College Station, TX, USA) in a 50 mm plastic petri dish every other day for six days to stimulate oogenesis. Adults were then provided 10 ml of bovine blood with 10 g of bovine liver (Rosenthal Meat Science and Technology Center, Texas A&M University, College Station, TX, USA) in a 50 mm plastic petri dish. After 48 h, 50 g of bovine liver in an 89 mL plastic bathroom cup (Walmart, Bentonville, AR, USA) was provided to the adults for oviposition. Adults had access to the bovine liver for 24 h, then the 50 g of bovine liver was replaced with fresh liver for subsequent egg clutches. Each day, liver and eggs were transferred into a 0.94 L wide mouth mason jar (Ball, Broomfield, CO, USA) filled half full of vermiculite (Producer's Cooperative Association, Bryan, TX, USA) for larval development. Larvae were provided additional fresh bovine liver *ad libitum*. Adult flies 7-9 d of age were used in the experiments.

Treatment Set Up

An hour prior to initiating a trial, the mice were placed in a biosafety cabinet in their original shipping containers. One at a time, each mouse was removed and placed on a sterile 10 x 15 cm piece of aluminum foil (Walmart, Bentonville, AR, USA). Each mouse carcass was swabbed with a single sterile cotton tipped applicator (Puritan Medical Products Company LLC, Guilford, ME, USA) wet with phosphate-buffered saline (PBS) on the left side (hind leg to neck and back) eight times to collect a skin microbiome sample.

The mouse was then transferred to a sterile 0.94 L wide mouth mason jar (Ball, Broomfield, CO, USA) with a 142 mm 0.2-micron filter (Sterlitech Corporation, Kent, WA, USA) on top, secured by the sterilized metal screw ring made for the jar (Ball, Broomfield, CO, USA). Control jars were also swabbed with a single sterile cotton tipped applicator before filter and metal screw ring placement. The cotton tipped applicators were placed in individual sterile 0.5 mL Eppendorf Microcentrifuge tubes (Eppendorf North America, Hauppaugue, NY, USA) at room temperature and transferred within an hour to a biosafety cabinet for further processing.

Treatment Verification

Each of the cotton swab samples were spread onto 100mm plates of Tryptic Soy Agar (TSA) (Neogen, Lansing, MI, USA) plates and TSA + 5% sheep blood plates (Blood, Dickinson, and Company, Franklin Lakes, NJ, USA) followed by incubation at 37°C for 24 h to ensure sterility on the controls and axenic mice. Any phenotypically distinct colonies were isolated and repeatedly subcultured onto fresh plates. The isolated bacteria were then cultured overnight on a blood plate at 37°C and identified via analysis with a Biolog Gen III Microstation (Biolog, Inc. Hayward, CA, USA).

Dual Choice Cube Olfactometer Assay

Methods were adapted from Flint and Tomberlin (Flint and Tomberlin 2020) (Figure 3.1). Before each use, olfactometer cubes and the PVC arms were cleaned with a 1% Alconox Powdered Precision Cleaner solution according to manufacturer's instructions (Alconox, Inc., White Plains, NY, USA) followed by 70% ethanol and

allowed to air dry. The PVC arms were further cleaned with acetone and allowed to air dry. Cube sleeves were washed with all® Free Clear liquid laundry detergent (Henkel Consumer Goods, New Province, NJ, USA) and allowed to air dry. Beginning at approximately 0800, ~200 flies (50:50 ♂:♀) were released into the center of each cube and allowed to acclimate for 30 min prior to exposure to treatments. Three treatment combinations were used: control (i.e., empty jar) and axenic mouse carcass, control and xenic mouse carcass, and axenic mouse carcass and xenic mouse carcass. Each treatment pair was randomly assigned to an olfactometer, and treatments were randomly assigned to the olfactometer arms. Based on results from a previous study, experiments were conducted at two- and eight-hour durations (Flint and Tomberlin 2020) after which, sticky traps were removed from the arms and new traps were placed inside the cube to collect the remaining flies. Flies collected on all sticky traps were sexed via interocular distance and the ovarian status was determined via dissection within 2 h to divide flies into males, non-gravid females, and gravid females (hereafter referred to as sex). This assay was performed with the same mouse carcasses after the mice were placed into the mason jar on days 0, 2, 4, 6, and 8. Three separate trials were conducted with two combination replicates each using succeeding generations of *C. macellaria* at 21°C.

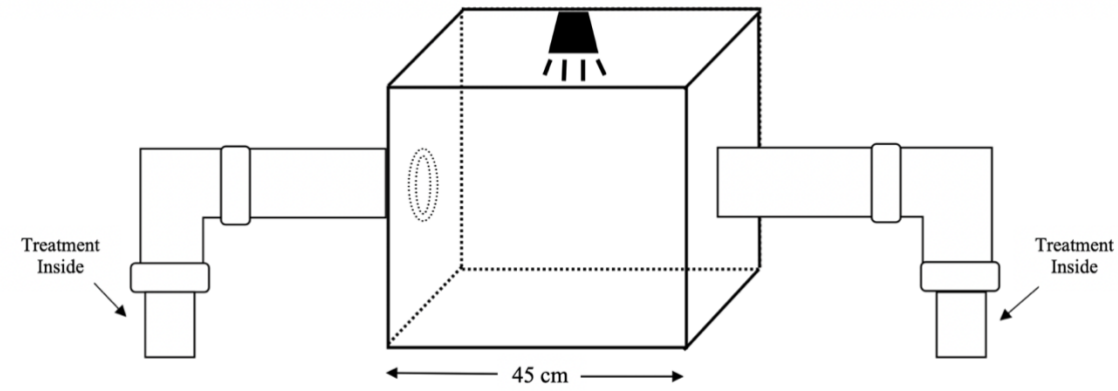


Figure 3.1. Diagram of the dual-choice cube olfactometer (Flint and Tomberlin 2020).

Volatile Organic Compound Set Up and Collection

These methods were adapted from other studies conducted on the same set up originally described by Beskin et al. (Beskin et al. 2018). Following the behavior assay, VOC samples were taken from each treatment jar at 21°C. Dual port bell jars were cleaned with a 1% Alconox Powdered Precision Cleaner solution according to manufacturer's instructions (Alconox, Inc., White Plains, NY, USA) followed by 95% ethanol and acetone and allowed to air dry. The bell jars were placed on top of each treatment jar and secured with Parafilm (Bemis Company, Inc, Neenah, WI, USA). The horizontal port was installed with a 14.6 cm glass Labcraft Pasteur pipet (Curtin Matheson Scientific, Inc., Morris Plains, NJ, USA) containing 0.75 g Black Diamond® activated carbon (Marineland, Cincinnati, OH, USA) with a 0.2-micron filter to purify incoming air secured by Parafilm. A volatile trap containing 30.0 mg of Hayesep® Q porous polymer (Volatile Assay Systems, Rensselaer, NY, USA) was placed in the vertical port to collect the VOCs

emitted by the treatments. The volatile traps were connected to the intake port on a flow meter (Dwyer Instruments, Inc., Michigan City, IN, USA) with 6.4 mm diameter Tygon® tubing (Saint-Gobain S.A., Malvern, PA, USA) secured by Parafilm. The exhaust port was attached to an AC110V, 60 Hz Rocker 300 oil free vacuum pump (Rocker, Scientific Co., Ltd., New Taipei City, Taiwan) with Tygon® tubing. VOC samples were allowed to accumulate in the set-up for one hour, after which the pump was turned on. Collections were made at a flow rate of 1 L min-1 for one hour. VOC samples were collected after each behavior assay on days 0, 2, 4, 6, and 8 from each mouse carcass and control.

Volatile Organic Compound Elution and Analysis

VOCs were eluted from the volatile trap into a 9 mm 375 µl insert (Thermo Fisher Scientific, Waltham, MA, USA) inside of a 2 mL SureStop ™ GC-MS vial (Thermo Fisher Scientific, Waltham, MA, USA) using 150 µl of dichloromethane (DCM) (Thermo Fisher Scientific, Waltham, MA, USA) and N_2. An internal standard consisting of 5 µl of 80 ng/µl n-Octane (Sigma-Aldrich, St. Louis, MO, USA) was added to each sample. Samples were sealed and stored at -20°C until analysis at the Geochemical and Environmental Research Group at Texas A&M University, College Station, TX, USA. Samples were analyzed with a Hewlett-Packard 7693 autosampler (Hewlett-Packard Company, Palo Alto, CA, USA) with a Hewlett-Packard 6890 gas chromatograph (Hewlett-Packard Company, Palo Alto, CA, USA) and paired with a Hewlett-Packard 5973 mass selective detector (Hewlett-Packard Company, Palo Alto, CA, USA). A DB-5 capillary column (30 m × 0.25 mm x 0.50 µm thick film) was used to separate the VOCs (Agilent Technologies, Santa Clara, CA, USA).

Injections of 1 µl were performed using a splitless mode using helium as a carrier gas (1.3 ml min-1) with an injection temperature of 250°C. The temperature ramp was as follows: an initial temperature of 35°C was held for 8 min, increase by 4°C min-1 until 60°C, increase by 6°C min-1 until 160°C, and finally, increase by 20°C min-1 until 300°C was attained. The mass selective detector had a 3 min solvent delay and mass range from m/z 50-550. The CG-MS data was processed using Chemstation software (Agilent Technologies, Santa Clara, CA, USA). Compounds were compared to those in the NIST 14 MS library of mass spectral database (Palisade Corp., Newfield, NY, USA).

Oviposition Assay

After the conclusion of the previous behavior and VOC collection steps (on day 8), all screw rings and 0.2-micron filters were removed, leaving the treatments exposed in the 0.94 L wide mouth mason jars. All treatment jars were randomly assigned to a corner in a 61 x 61 x 61 cm BioQuip Lumite Screen Collapsible Cage (BioQuip Inc., CA, USA) which contained approximately 2000 (50:50 ♂: ♀) 7-9-d-old adult *C. macellaria* reared according to the previous section. After 24 h, treatment jars were removed and egg masses present were collected using a fine-tip paint brush, placed in a weigh boat, and weighed on an Ohaus Adventurer™ Pro (Ohaus, Parsippany, NJ, USA) scale to calculate the egg mass weight. As with previous experiments, four replicates were conducted per trial with 5 trials completed.

Statistical Analysis

All data were analyzed using RStudio® version 1.2.1335 (Team 2013). For the dual-choice cube olfactometer assay, normality and equal variances were tested using a Shapiro-Wilk and Bartlett test, respectively. An analysis of variance (ANOVA) was conducted with an alpha of 0.05 to determine if the level of response varied over time (days of the experiment) within a treatment in addition to differences between treatment responses during a single time point. All assumptions were met unless otherwise stated and non-significant interactions were removed from analyses. Due to the successional nature of the behavior trials, two- and eight-hour data were not compared. Each treatment pair was analyzed independently. To determine and visualize differences in volatile organic compound profiles, non-metric multidimensional scaling (NMDS) with Bray-Curtis dissimilarity matrix and 999 permutations was implemented. A permutational multivariate analysis of variance (PERMANOVA) was used to identify differences in treatments, days, and their interaction. Pairwise comparisons were then completed to identify significant differences between treatments and days using an alpha of 0.05. An indicator species analysis (ISA) was performed to identify indicator compounds associated with a treatment or group of treatments. Mean species richness (abundance), evenness, and Shannon-Wiener diversity indices (H) were calculated over time, with a repeated measures analysis of variance (RM-ANOVA) to detect significant changes in treatments over time. Oviposition data met the assumptions of a parametric test unless otherwise stated. A t-test was used to determine the significance of microbial presence and absence on oviposition preference. As with other tests, the alpha was 0.05.

Results

Treatment Verification

All control jars remained sterile throughout the duration of the experiment except for one sample at the end of one trial. All axenic mice maintained their axenic status throughout the duration of the experiment except for approximately 1 mouse per trial and the contaminant was identified as *Micrococcus luteus*. Our findings indicate that microbial contamination did not influence blow fly response.

Microbiome and Sex/Ovarian Development Impact on Attraction

Each data set was analyzed by total response (i.e., departing the center cube as opposed to making no choice and remaining in the center cube) and treatment choice of the responders (i.e., choosing one arm as opposed to the other). Each set was analyzed as pooled sex data and then by sex (e.g., male and female) and physiological state (e.g., gravid and non-gravid).

2 hour – Control v Axenic Total Response – Analysis of total fly response based on pooled sex and physiological state to the control as well as the axenic mouse (treatment) rather than remain in the cube had a significant ($df = 14, 15$; $F = 10.28$; $p < 0.001$) trial*day interaction. Trial 2 had approximately 4.5% greater response compared to trials 1 and 3; however, order of response level to either treatment-control combination or not to respond was consistent. Overall response ranged from ~11% to ~21% throughout the experiment (Figure 3.2A) with no discernable pattern over time.

When analyzed by sex and physiological state, the data were not normally distributed and therefore were square root transformed. A significant ($df = 44, 45$; $F = 12.995$; $p < 0.001$) three-way interaction between day, trial, and sex for predicting level of response to the axenic mouse was determined. In general, males responded twice as much as gravid and non-gravid females (Figure 3.2B).

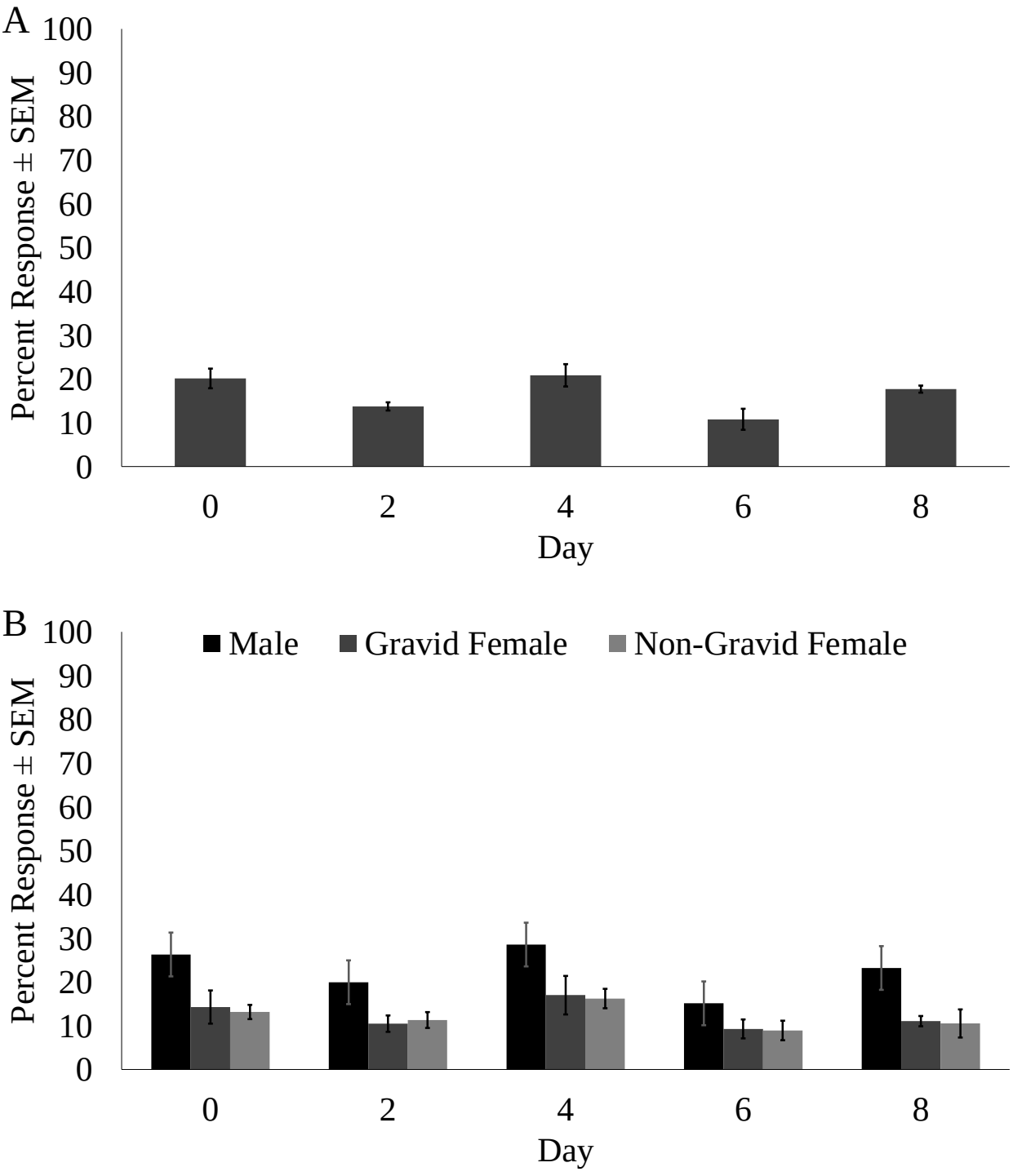
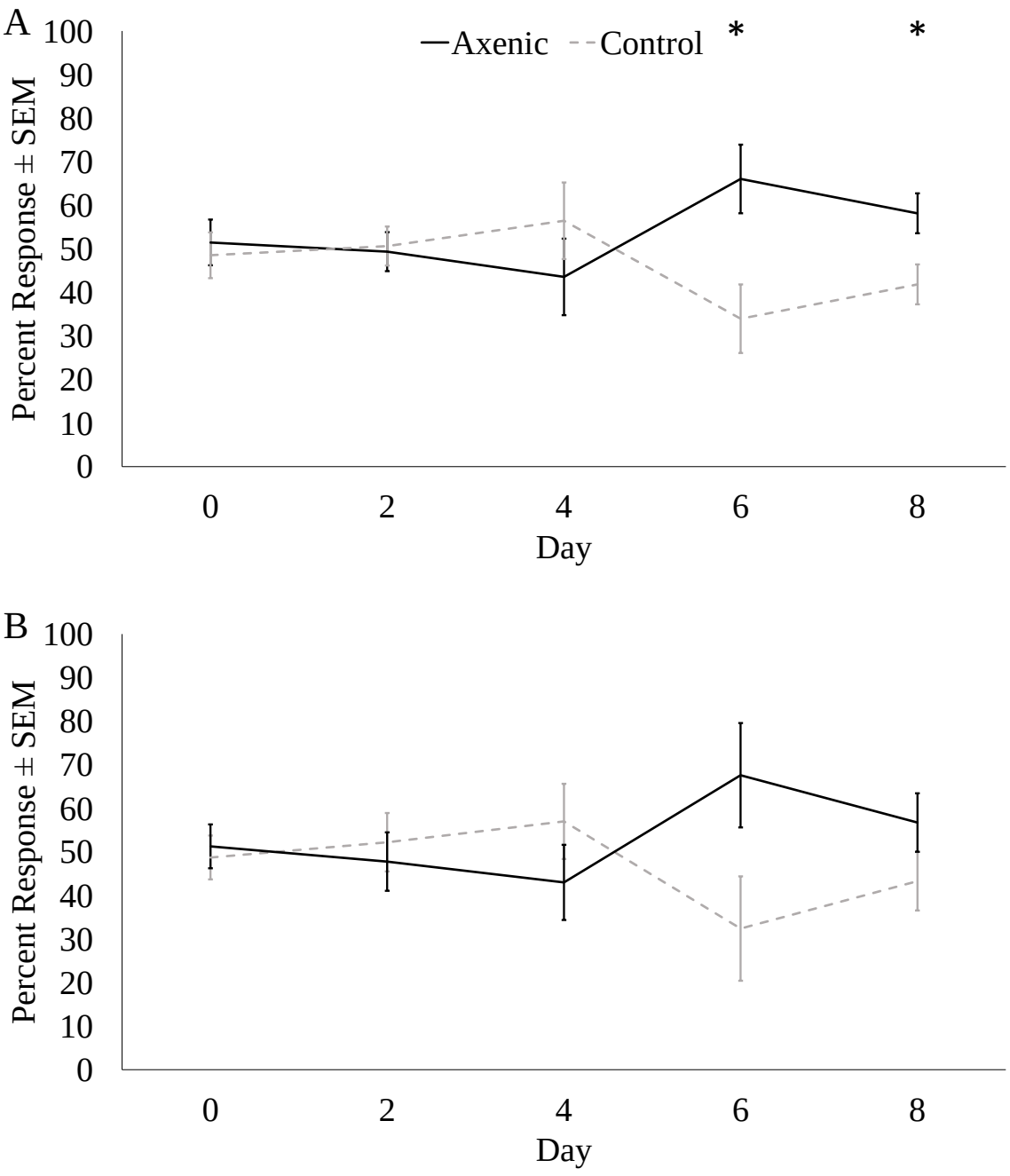


Figure 3.2. Average percent ± SEM response of ~200 7-9 d old male, gravid, and non-gravid *C. macellaria* in an olfaction cube maintained at approximately 21°C to axenic mouse and control treatments for (A) overall response with a pooled sex and (B) overall response by sex in 2-hour trials.

2 hour – Control v Axenic Treatment Response - No significant interactions ($p > 0.05$) for fly response based on pooled sex and physiological state to the axenic mouse (treatment) as opposed to the blank control were discovered and therefore were removed from the analysis. Subsequent analysis demonstrated the level of fly response based on pooled sex and physiological state to the axenic mouse did not significantly differ across trial ($df = 2, 23$; $F = 1.237$; $p = 0.309$) or day ($df = 4, 23$; $F = 1.832$; $p = 0.157$) (Figure 3.3A). In totality, flies responded more to the axenic mouse than the control late in the decomposition process on days 6 (~33%; $df = 1, 10$; $F = 8.302$; $p = 0.016$) and 8 (~17%; $df = 1, 10$; $F = 6.33$; $p = 0.03$) (Figure 3.3A).

When treatment choice was analyzed by sex and physiological state, fly response to the axenic mouse was not significantly ($p > 0.05$) impacted by interactions and were therefore removed. Subsequent analysis demonstrated the level of fly response to the axenic mouse was not impacted by sex ($df = 2, 81$; $F = 0.052$; $p = 0.949$) or trial ($df = 2, 81$; $F = 1.618$; $p = 0.204$) but was impacted by day ($df = 4, 81$; $F = 3.268$; $p = 0.015$). Comparison of fly response to the axenic mouse versus the control revealed that gravid females on day 8 (~14%; $df = 1, 10$; $F = 39.22$; $p < 0.001$) and non-gravid females on day 6 (~53%; $df = 1, 10$; $F = 17.63$; $p = 0.001$) significantly preferred the axenic mouse over the control (Figure 3.3C and 3.3D). Although not statistically significant (~35%; $df = 1, 10$; $F = 4.312$; $p = 0.064$), males presented a similar preference for the axenic mouse on day 6 (Figure 3.3B).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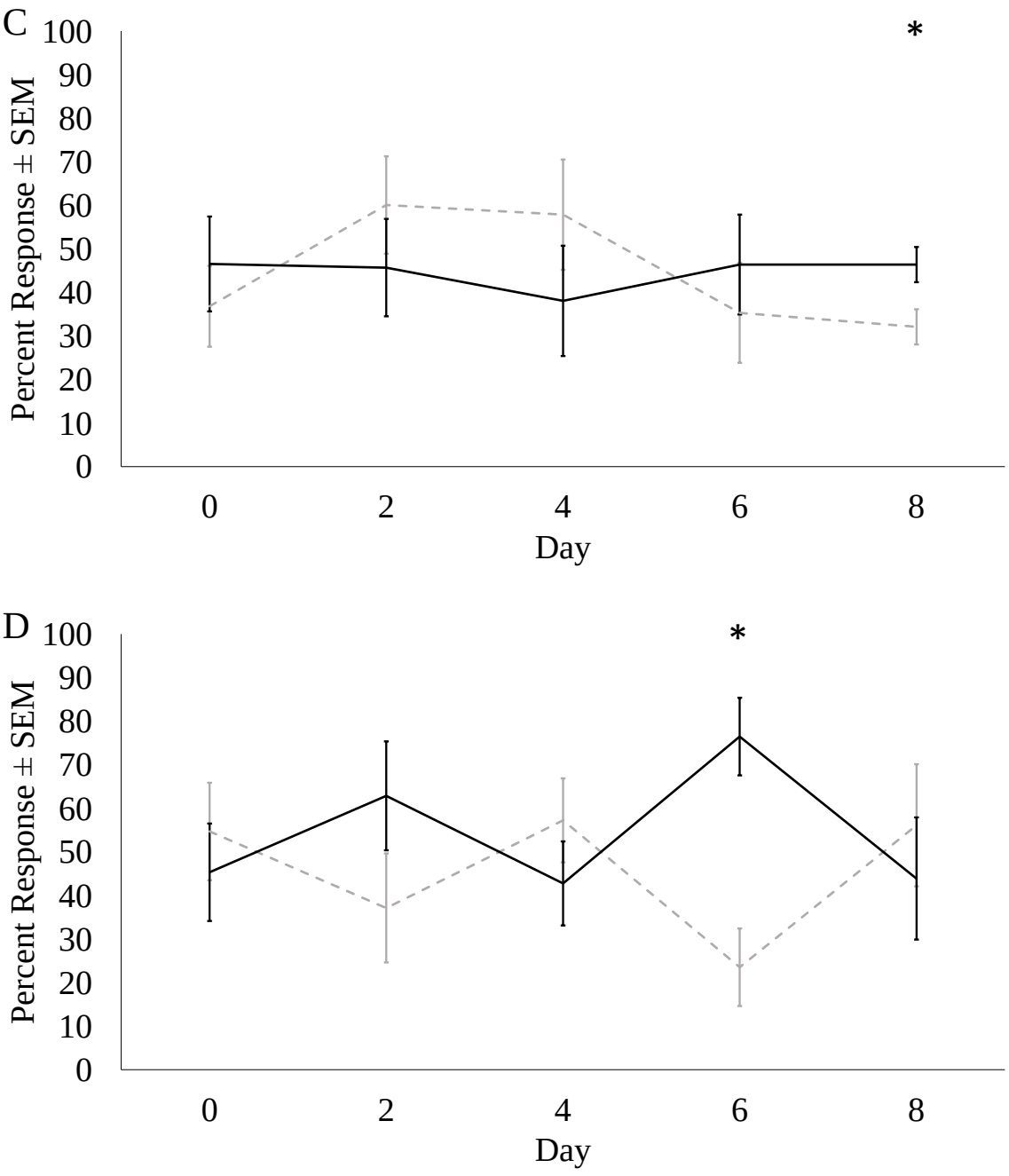


Figure 3.3. Average percent ± SEM response of ~200 7-9 d old *C. macellaria* in an olfaction cube maintained at approximately 21°C to an axenic mouse treatment and control (empty jar) for (A) pooled sex, (B) males, (C) gravid females, and (D) non-gravid females in 2-hour trials; [1]* indicates significance of $p < 0.05$.

2 hour – Control v Xenic Total Response – Analysis of total fly response based on pooled sex and physiological state to the control as well as the xenic mouse (treatment) rather than remain in the cube did not have a significant ($p > 0.05$) trial*day interaction and was removed from analysis. Subsequent analysis revealed trial ($df = 2, 23$; $F = 12.26$; $p < 0.001$) and day ($df = 4, 23$; $F = 3.256$; $p = 0.029$) were significant in predicting level of response for pooled sex and physiological state data. Trial 2 had approximately 7.5% greater response compared to trials 1 and 3; however, order of response level to either treatment-control combination or not to respond was consistent. Level of overall response doubled from day 6 to day 8 (Figure 3.4A) but had no discernable pattern over time.

When analyzed by sex and physiological state, the data were not normally distributed and therefore were square root transformed and non-significant interactions removed from analysis. Sex ($df =2, 81$; $F = 17.003$; $p < 0.001$), trial ($df = 2, 81$; $F = 11.772$; $p < 0.001$), and day ($df = 4, 81$; $F = 3.258$; $p = 0.015$) significantly impact the level of response. In general, males responded significantly more than gravid and non-gravid females, up to responding twice as much (Figure 3.4B). Overall response on day 6 was significantly less than response on days 2, 4, and 8 (Figure 3.4B).

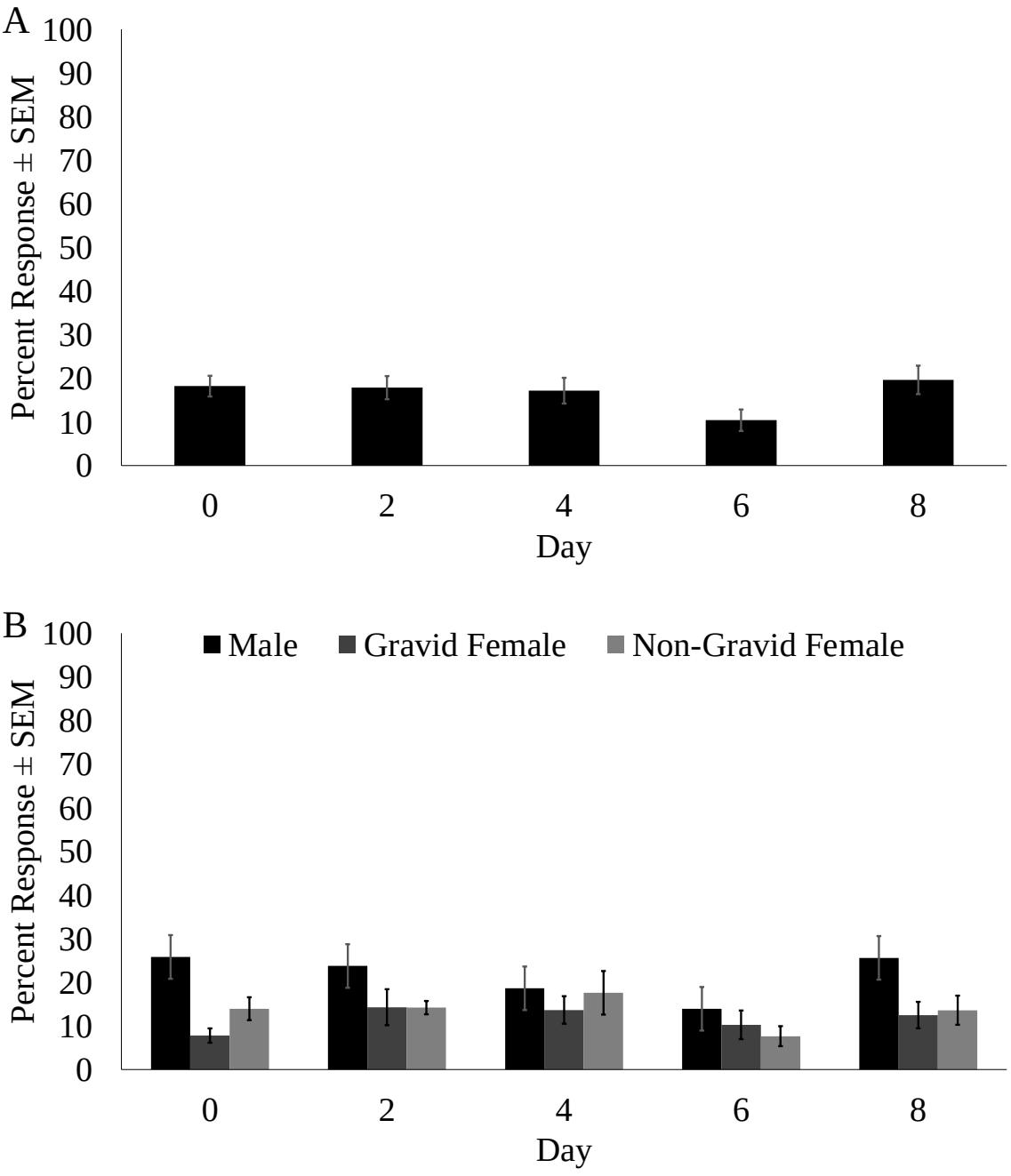
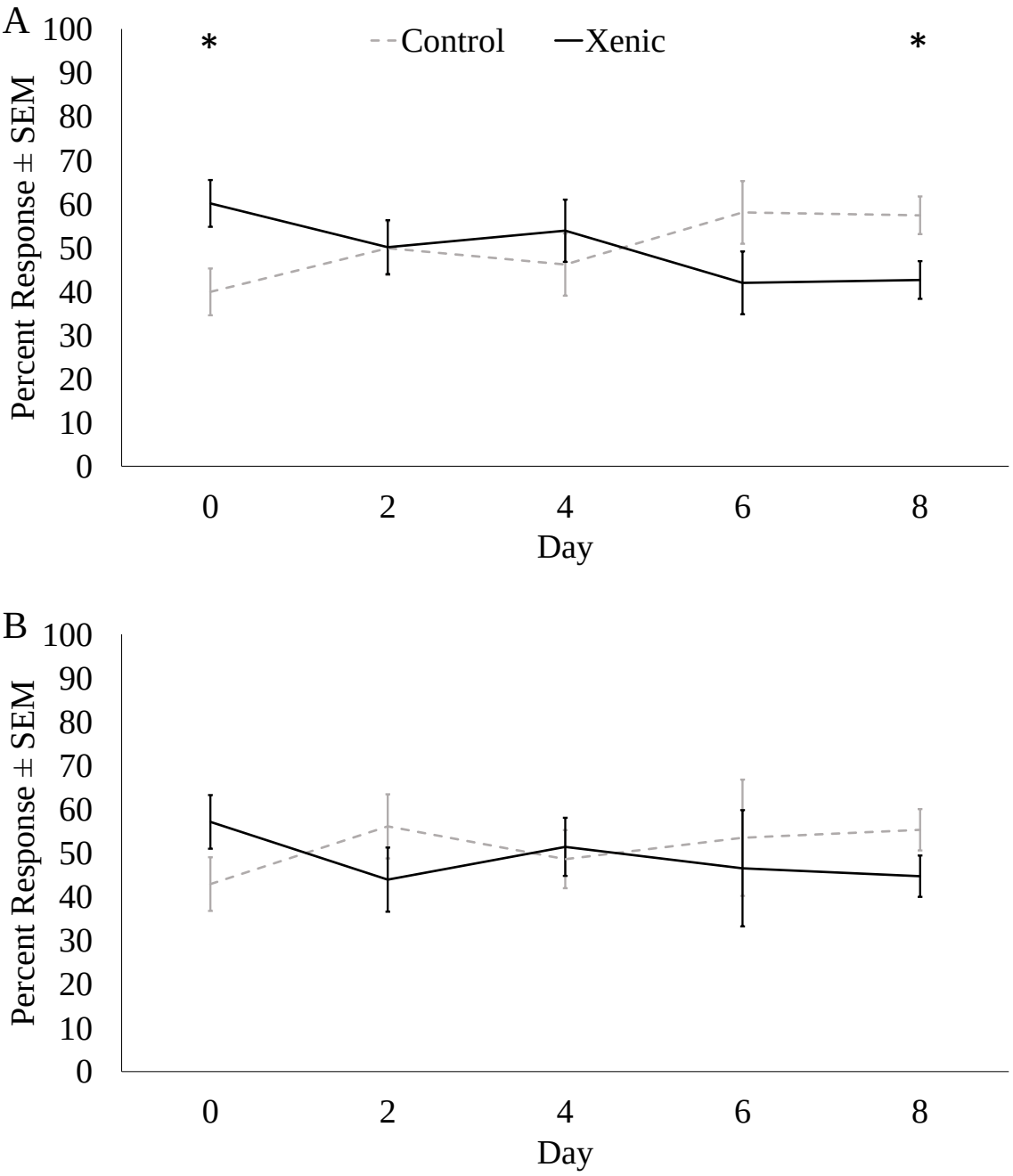


Figure 3.4. Average percent ± SEM response of ~200 7-9 d old male, gravid, and non-gravid *C. macellaria* in an olfaction cube maintained at approximately 21°C to xenic mouse and control treatments for (A) overall response with a pooled sex and (B) overall response by sex in 2-hour trials.

2 hour – Control v Xenic Treatment Response – Analysis of total fly response based on pooled sex and physiological state to the xenic mouse (treatment) as opposed to the blank control had a significant ($df = 8, 15$; $F = 8.716$; $p < 0.001$) trial*day interaction. In totality, flies responded more to the xenic mouse than the control early in the decomposition process on day 0 (~20%; $df = 1, 10$; $F = 7.13$; $p = 0.023$) but preferred the control late in the decomposition process on day 8 (15%; $df = 1, 10$; $F = 5.892$; $p = 0.035$) (Figure 3.5A).

When treatment choice was analyzed by sex and physiological state, fly response to the xenic mouse was not significantly ($p > 0.05$) impacted by a trial*day*sex interaction and was removed. Subsequent analysis demonstrated the level of fly response to the xenic mouse was not impacted by sex ($df = 2, 60$; $F = 0.823$; $p = 0.444$), day ($df = 4, 60$; $F = 2.209$; $p = 0.078$), trial ($df = 2, 60$; $F = 2.122$; $p = 0.128$), sex*day ($df = 8, 60$; $F = 1.583$; $p = 0.149$), and sex*trial ($df = 4, 60$; $F = 0.356$; $p = 0.838$) but was impacted by trial*day ($df = 8, 60$; $F = 3.617$; $p = 0.001$). Comparison of fly response to the xenic mouse versus the control revealed that non-gravid females preferred the xenic mouse on day 0 (~46%; $df = 1, 10$; $F = 15.7$; $p = 0.002$) but preferred the control on day 6 (~37%; $df = 1, 10$; $F = 5.542$; $p = 0.04$) (Figure 3.5D). In general, males tended to prefer the control (Figure 3.5B) while gravid females preferred the xenic mouse with the exception of day 8 (Figure 3.5C). Although not statistically significant (35%; $df = 1, 10$; $F = 4.138$; $p = 0.069$), gravid females had the highest attraction to the xenic mouse on day 2 compared to the control (Figure 3.5C).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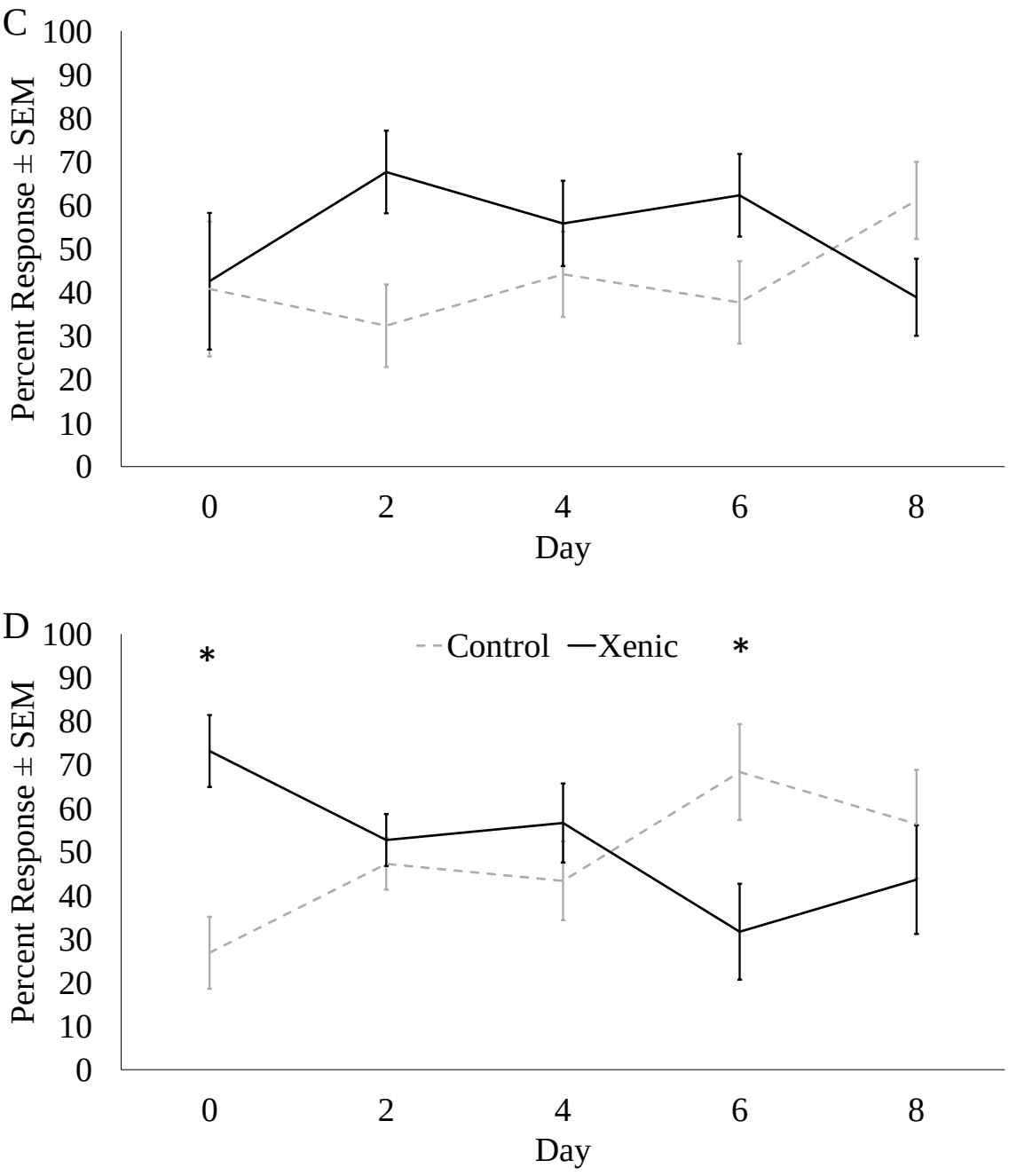


Figure 3.5. Average percent ± SEM response of ~200 7-9 d old *C. macellaria* in an olfaction cube maintained at approximately 21°C to a xenic mouse treatment and control (empty jar) for (A) pooled sex, (B) males, (C) gravid females, and (D) non-gravid females in 2-hour trials; [1]* indicates significance of $p < 0.05$.

2 hour – Axenic v Xenic Total Response – Analysis of total fly response based on pooled sex and physiological state to the axenic moue (treatment) as well as the xenic mouse (treatment) rather than remain in the cube had a significant (df = 14, 15; F = 4.248; p = 0.004) trial*day interaction. Trial 2 had approximately 7.7% greater response compared to trials 1 and 3; however, order of response level to either treatment combination or not to respond was consistent. Overall response ranged from ~13% to ~22% throughout the experiment (Figure 3.6A) with no discernable difference over time.

When analyzed by sex and physiological state, the data were not normally distributed and therefore were square root transformed. A significant (df = 44, 45; F = 2.186; p = 0.005) three-way interaction between day, trial, and sex for predicting level of response was determined. In general, males responded significantly more than gravid and non-gravid females, up to responding twice as much and peak female (gravid and non-gravid) response was on day 4 (Figure 3.6B).

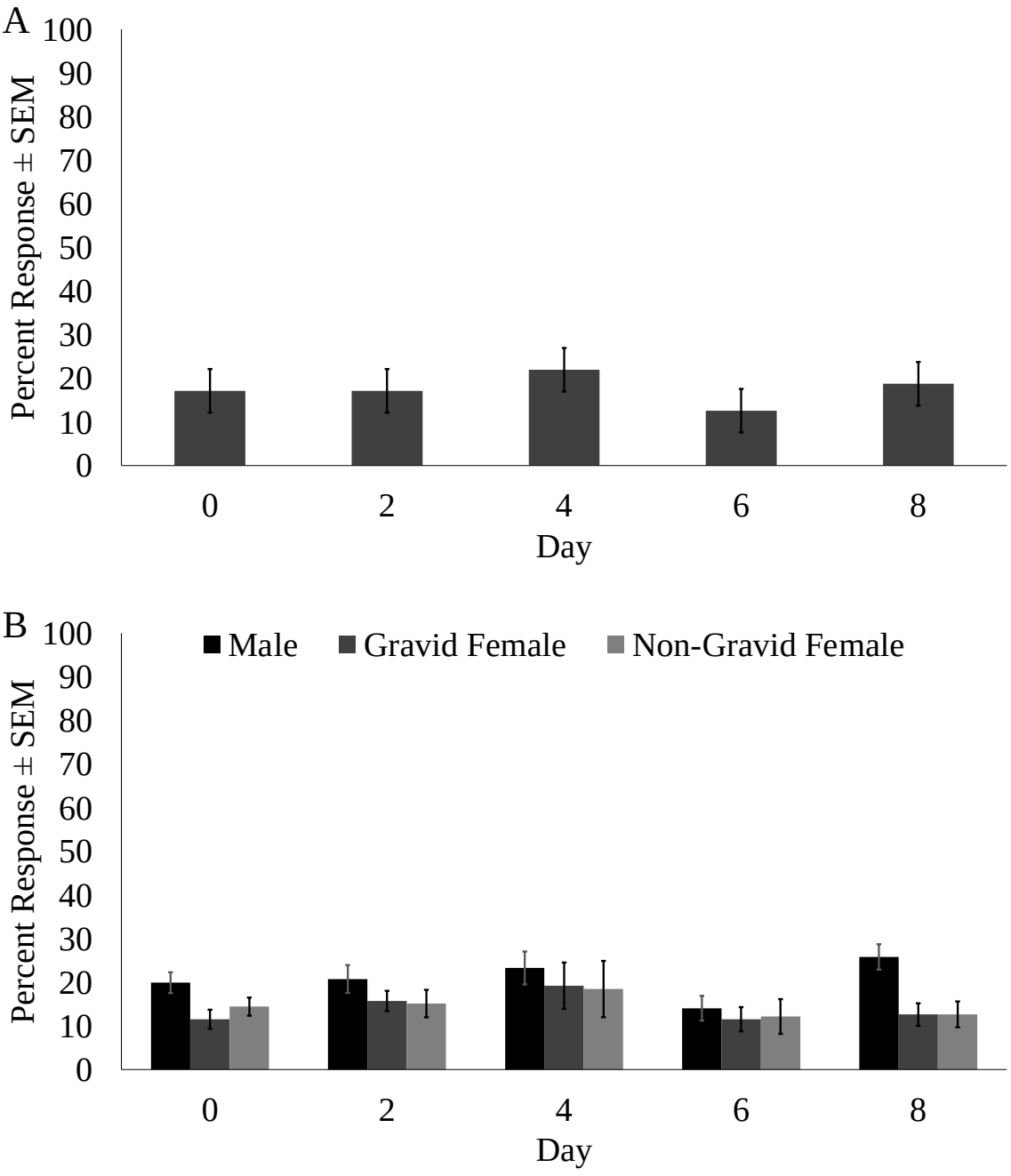
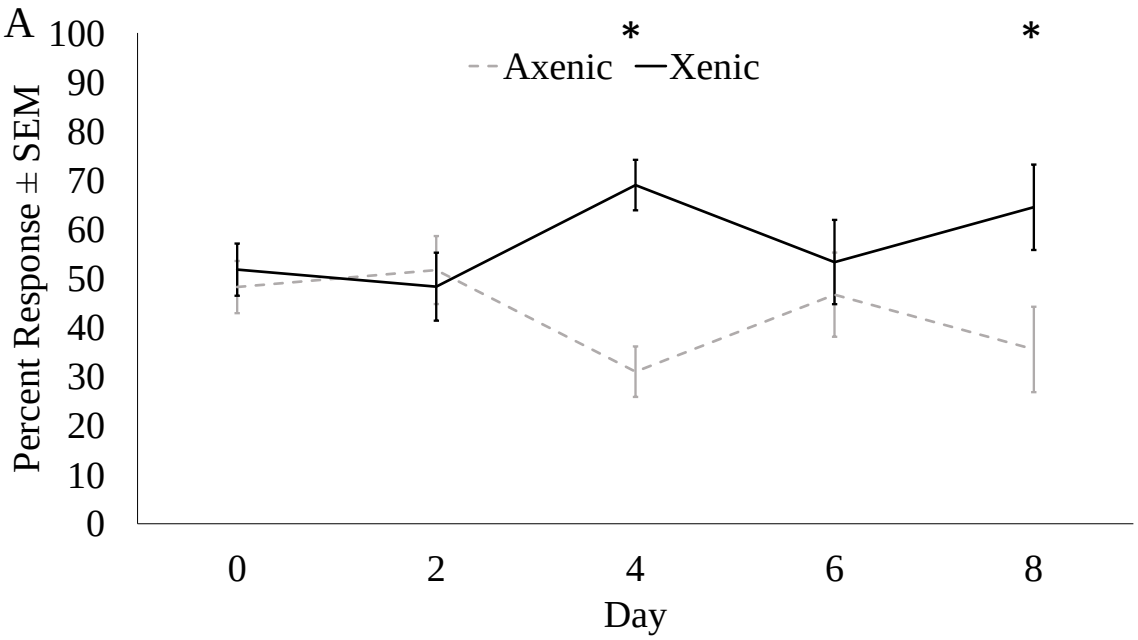


Figure 3.6. Average percent ± SEM response of ~200 7-9 d old male, gravid, and non-gravid *C. macellaria* in an olfaction cube maintained at approximately 21°C to xenic mouse and axenic mouse treatments for (A) overall response with a pooled sex and (B) overall response by sex in 2-hour trials.

2 hour – Axenic v Xenic Treatment Response – No significant interactions ($p > 0.05$) for fly response based on pooled sex and physiological state to the axenic mouse (treatment) as opposed to the xenic mouse (treatment) were discovered and therefore were removed from the analysis. Subsequent analysis demonstrated the level of fly response based on pooled sex and physiological state to the axenic mouse did not significantly differ across trial ($df = 2, 23$; $F = 1.807$; $p = 0.187$) or day ($df = 4, 23$; $F = 1.667$; $p = 0.192$) (Figure 3.7A). In totality, flies responded more to the xenic mouse than the axenic mouse later in the decomposition process on day 4 (~39%; $df = 1, 10$; $F = 27.31$; $p < 0.001$) and day 8 (~29%; $df = 1, 10$; $F = 5.539$; $p = 0.040$) (Figure 3.7A).

When treatment choice was analyzed by sex and physiological state, fly response to the axenic mouse was not significantly ($p > 0.05$) impacted by a trial*day*sex interaction and was removed. Subsequent analysis demonstrated the level of fly response to the axenic mouse was not impacted by sex ($df = 2, 80$; $F = 0.247$; $p = 0.781$) but was impacted by day ($df = 4, 80$; $F = 2.935$; $p = 0.025$) and trial ($df = 2, 80$; $F = 4.944$; $p = 0.009$). Comparison of fly response to the axenic mouse versus the xenic mouse revealed that non-gravid females preferred the axenic mouse early in the decomposition process on day 2 (~21%; $df = 1, 10$; $F = 5.75$; $p = 0.037$) but preferred the xenic mouse later in the decomposition process on days 4 (~40%; $df = 1, 10$; $F = 7.619$; $p = 0.024$) and 8 (~50%; $df = 1, 10$; $F = 10.81$; $p = 0.008$) (Figure 3.7D). On day 4, males (~39%; $df = 1, 10$; $F = 29.88$; $p < 0.001$) and gravid females (~43%; $df = 1, 10$; $F = 17.6$; $p = 0.001$) also preferred the xenic mouse over the axenic mouse (Figure 3.7B and 3.7C).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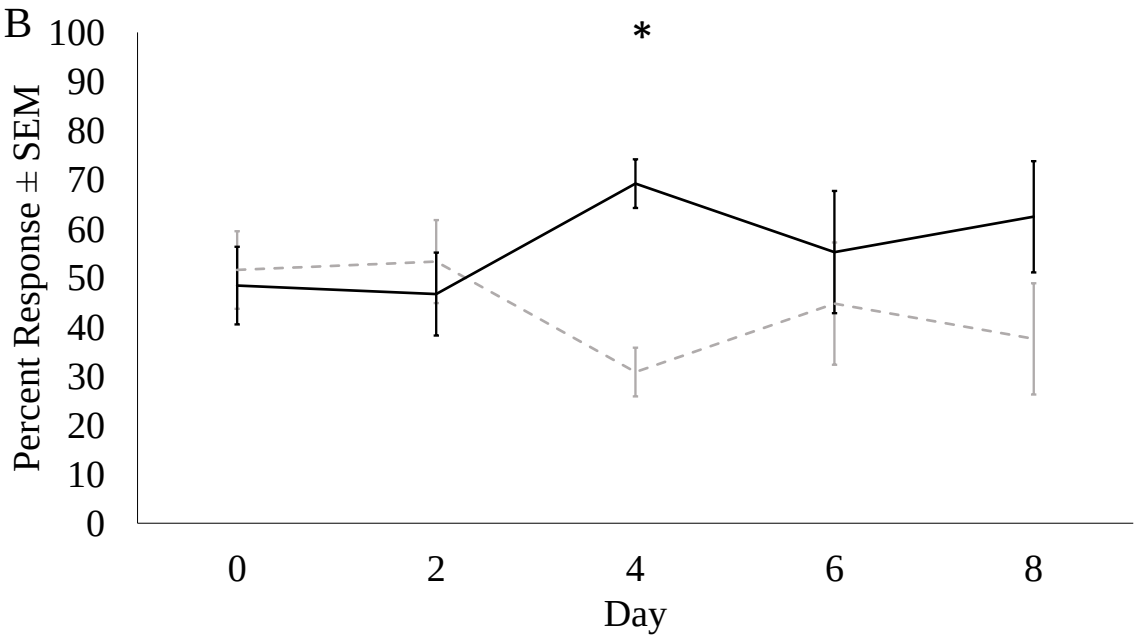
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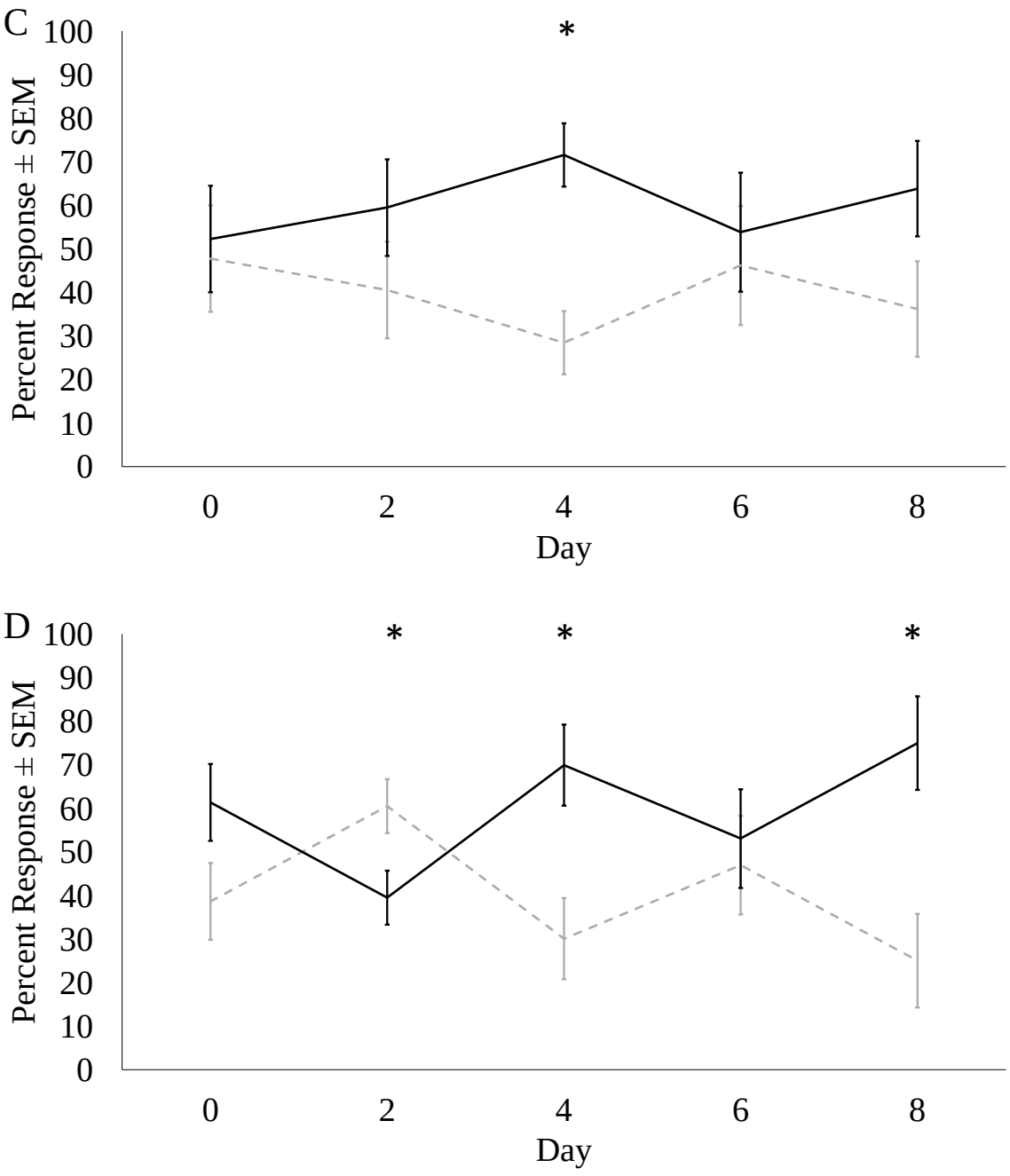


Figure 3.7. Average percent ± SEM response of ~200 7-9 d old *C. macellaria* in an olfaction cube maintained at approximately 21°C to xenic mouse and axenic mouse treatments for (A) pooled sex, (B) males, (C) gravid females, and (D) non-gravid females in 2-hour trials; [1]* indicates significance of $p < 0.05$.

8 hour – Control v Axenic Total Response – Analysis of total fly response based on pooled sex and physiological state to the control as well as the axenic mouse (treatment) rather than remain in the cube had a significant ($df = 14, 15$; $F = 5.795$; $p < 0.001$) trial*day interaction. There was no discernable pattern of order of response level to either treatment-control combination or not to respond. Overall response ranged from ~27% to ~39% throughout the experiment (Figure 3.8A) with an increase and then plateau in level of response over time.

When analyzed by sex and physiological state, the data were not normally distributed and therefore were square root transformed. A significant ($df = 44, 45$; $F = 4.388$; $p < 0.001$) three-way interaction between day, trial, and sex for predicting level of response was determined. In general, males responded more than gravid and non-gravid females ranging from an 8% difference to a 14% difference with the exception of days 0 and 6 where gravid females had the highest level of response (Figure 3.8B).

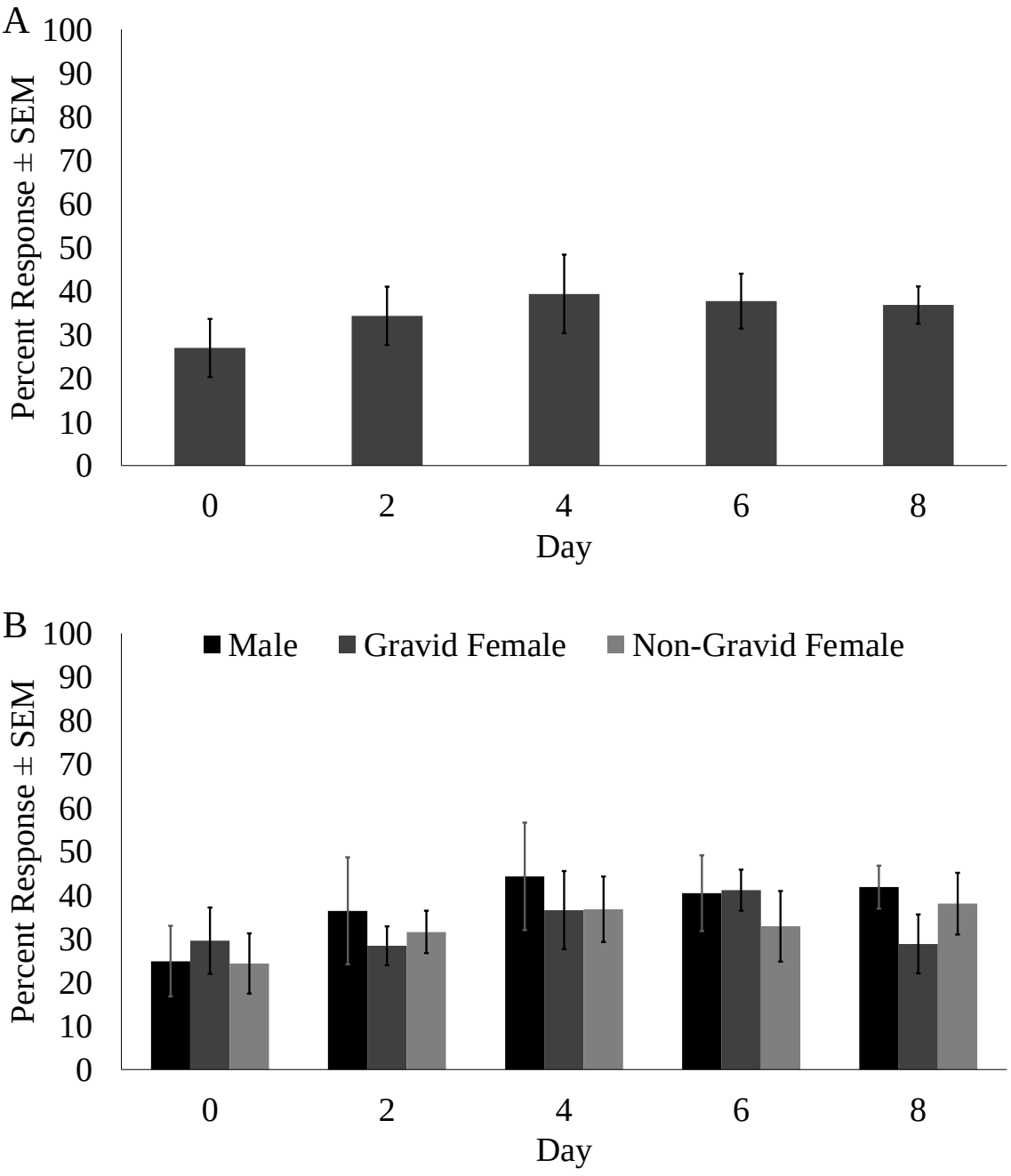
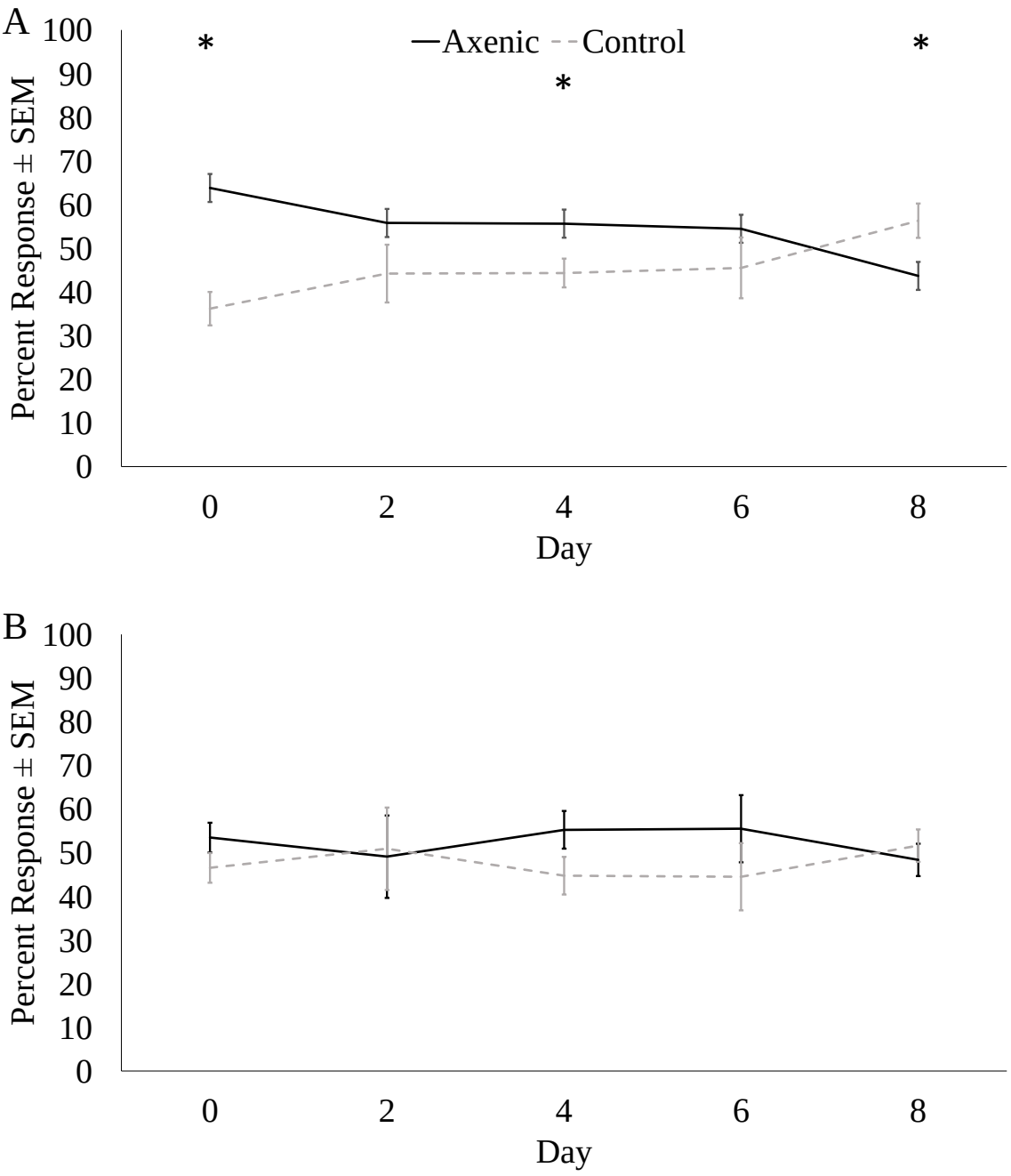


Figure 3.8. Average percent ± SEM response of ~200 7-9 d old male, gravid, and non-gravid *C. macellaria* in an olfaction cube maintained at approximately 21°C to axenic mouse and control treatments for (A) overall response with a pooled sex and (B) overall response by sex in 8-hour trials.

8 hour – Control v Axenic Treatment Response – No significant interactions ($p > 0.05$) for fly response based on pooled sex and physiological state to the axenic mouse (treatment) as opposed to the blank control were discovered and therefore were removed from the analysis. Subsequent analysis demonstrated the level of fly response based on pooled sex and physiological state to the axenic mouse did not significantly differ across trial ($df = 2, 23$; $F = 0.330$; $p = 0.722$) or day ($df = 4, 23$; $F = 1.834$; $p = 0.157$). In totality, flies responded more to the axenic mouse compared to the control early in the decomposition process on days 0 (~28%; $df = 1, 10$; $F = 25.99$; $p < 0.001$) and 4 (~12%; $df = 1, 10$; $F = 5.882$; $p = 0.0357$) but preferred the control later in the decomposition process on day 8 (~13%; $df = 1, 10$; $F = 5.157$; $p = 0.0465$) (Figure 3.9A).

When treatment choice was analyzed by sex and physiological state, fly response to the axenic mouse was not significantly ($p > 0.05$) impacted by a trial*day*sex interaction and was removed. Subsequent analysis demonstrated the level of fly response to the axenic mouse was not impacted by sex ($df = 2, 80$; $F = 0.354$; $p = 0.703$) or trial ($df = 2, 80$; $F = 0.277$; $p = 0.759$) but was impacted by day ($df = 4, 80$; $F = 3.501$; $p = 0.011$). Comparison of fly response to the axenic mouse versus the control revealed that gravid females preferred the axenic mouse early in the decomposition process on day 0 (~36%; $df = 1, 10$; $F = 17.62$; $p = 0.001$), day 2 (~34%; $df = 1, 10$; $F = 6.648$; $p = 0.027$), and day 4 (~15%; $df = 1, 10$; $F = 5.545$; $p = 0.040$) (Figure 3.9C). Non-gravid females preferred the axenic mouse early in the decomposition process on day 0 (~44%; $df = 1, 10$; $F = 38.97$; $p < 0.001$) but preferred the control later in the decomposition process on day 8

(~20%; df = 1, 10; F = 16.02; p = 0.002) (Figure 3.9D). Males had no preference between the axenic mouse or control for the duration of the experiment (Figure 3.9B).



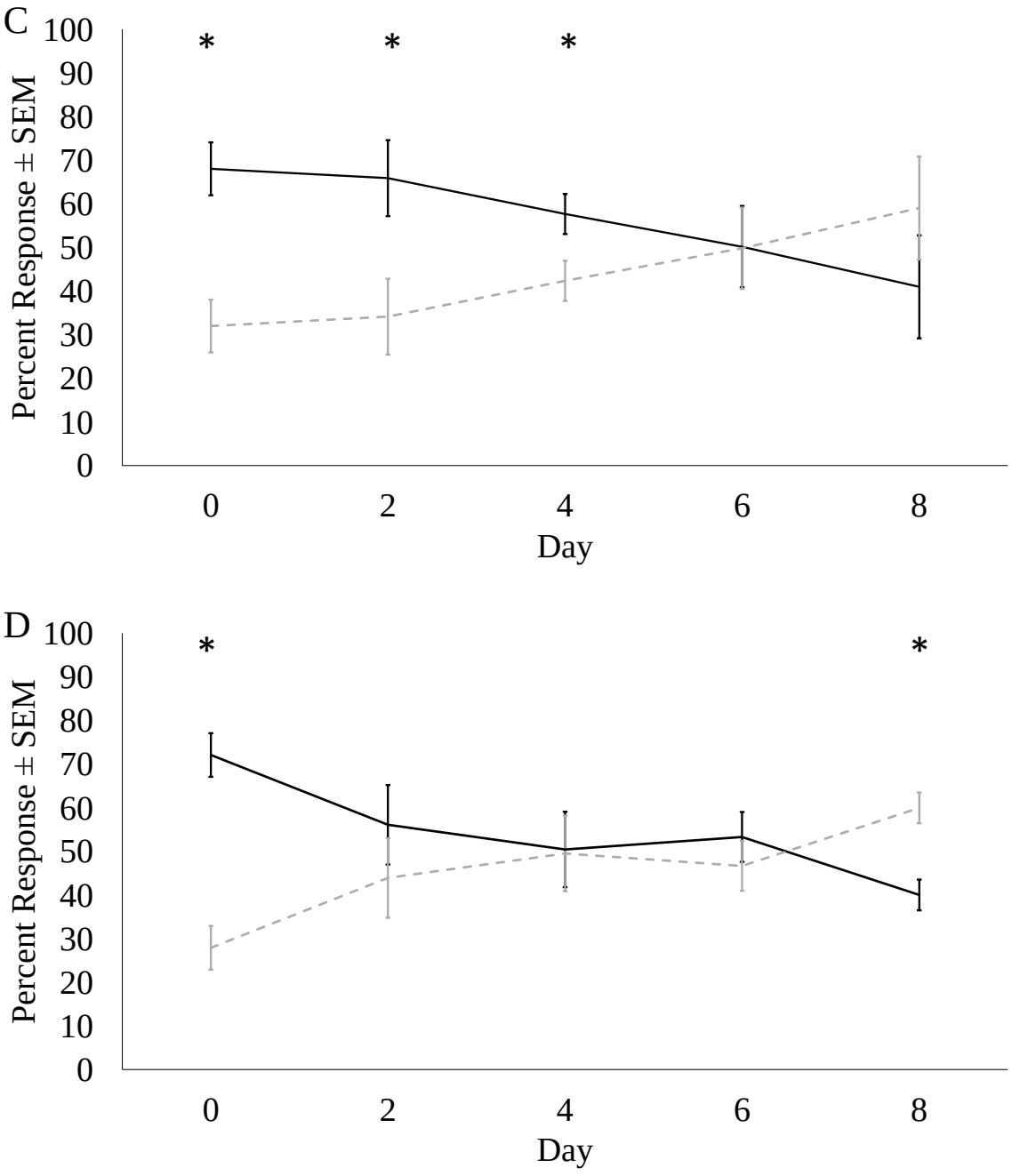


Figure 3.9. Average percent ± SEM response of ~200 7-9 d old *C. macellaria* in an olfaction cube maintained at approximately 21°C to an axenic mouse treatment and control (empty jar) for (A) pooled sex, (B) males, (C) gravid females, and (D) non-gravid females in 8-hour trials; [1]* indicates significance of $p < 0.05$.

8 hour – Control v Xenic Total Response – Analysis of total fly response based on pooled sex and physiological state to the control as well as the xenic mouse (treatment) rather than remain in the cube had a significant ($df = 14, 15$; $F = 9.205$; $p < 0.001$) trial*day interaction. There was no discernable pattern of order of response level to either treatment-control combination or not to respond. Overall response ranged from ~27% to ~41% throughout the experiment (Figure 3.10A) with an increase and then plateau in level of response over time.

When treatment choice was analyzed by sex and physiological state, fly response to the xenic mouse was not significantly (chi square = 0.528; $df = 2$; $p = 0.767$) impacted by trial. Sex*day ($df = 14, 75$; $F = 2.607$; $p = 0.0234$) significantly impacted level of response to the xenic mouse. Overall, males responded more than gravid and non-gravid females later in the decomposition process on days 6 and 8 while gravid females responded more than males and non-gravid females early in the decomposition process on days 2 and 4 (Figure 3.10B).

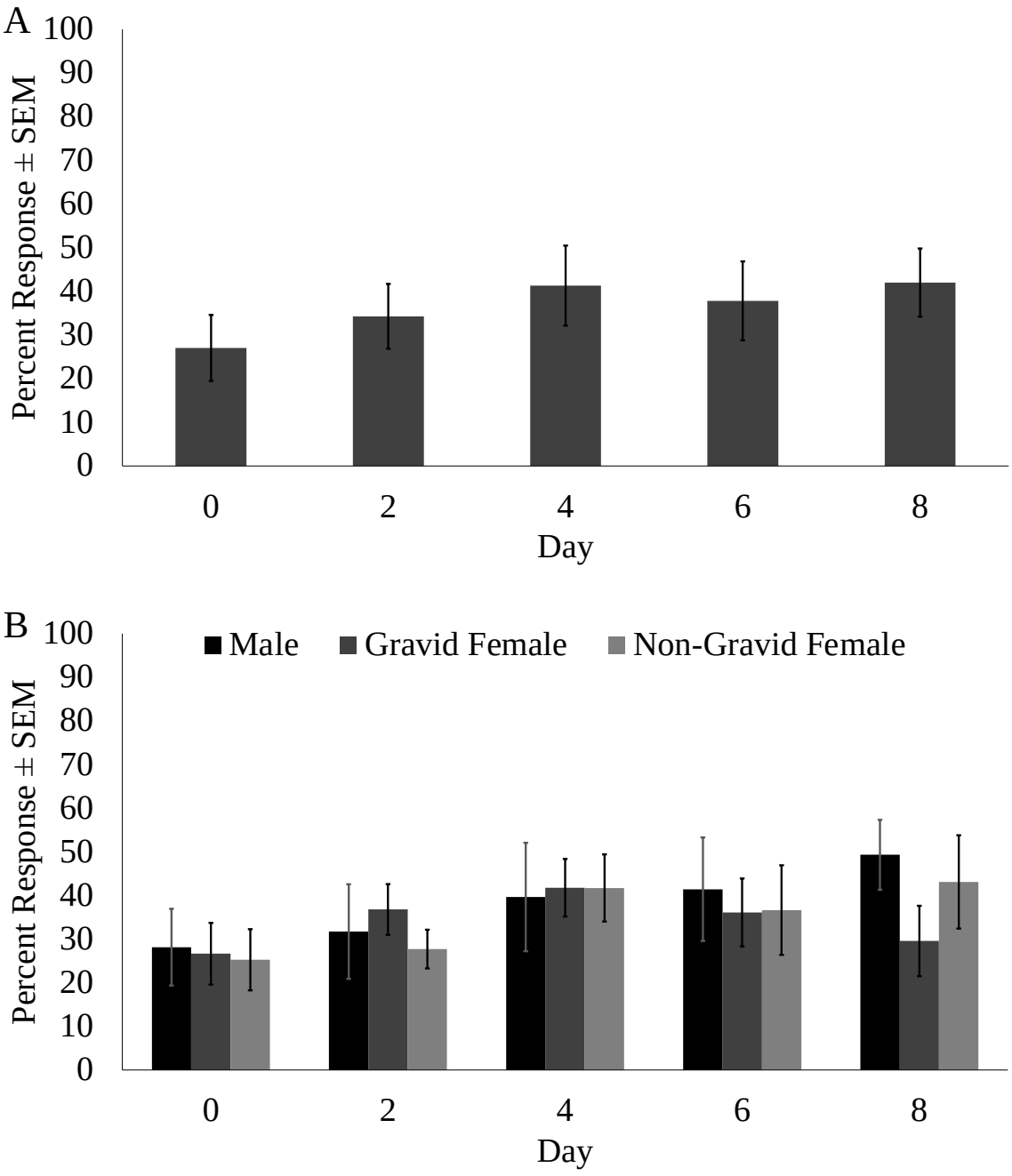
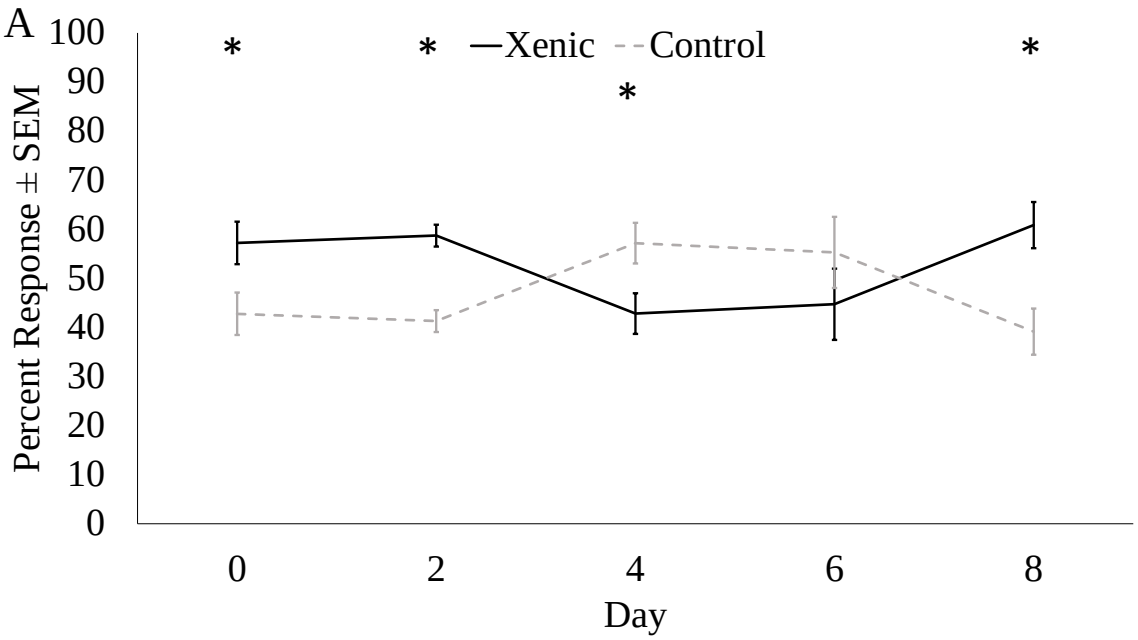


Figure 3.10. Average percent ± SEM response of ~200 7-9 d old male, gravid, and non-gravid *C. macellaria* in an olfaction cube maintained at approximately 21°C to xenic mouse and control treatments for (A) overall response with a pooled sex and (B) overall response by sex in 8-hour trials.

8 hour – Control v Xenic Treatment Response – No significant interactions ($p > 0.05$) for fly response based on pooled sex and physiological state to the xenic mouse (treatment) as opposed to the blank control were discovered and therefore were removed from the analysis. Subsequent analysis demonstrated the level of fly response based on pooled sex and physiological state to the xenic mouse did not significantly differ across trial ($df = 2, 23$; $F = 0.424$ $p = 0.659$) but do differ across day ($df = 4, 23$; $F = 2.929$; $p = 0.042$). In totality, flies responded more to the xenic mouse compared to the control early in the decomposition process on days 0 (~15%; $df = 1, 10$; $F = 5.547$; $p = 0.040$) and 2 (~17%; $df = 1, 10$; $F = 30.55$; $p < 0.001$) and later in the decomposition process on day 8 (~23%; $df = 1, 10$; $F = 10.64$; $p = 0.008$), but preferred the control in the middle of the decomposition process on day 4 (~13%; $df = 1, 10$; $F = 5.99$; $p = 0.034$) (Figure 3.11A).

When treatment choice was analyzed by sex and physiological state, level of response to the xenic mouse was not significantly impacted by three-way or two-way interactions ($p > 0.05$) and were removed. Subsequent analysis reveals sex ($df = 2, 81$; $F = 0.1.532$; $p = 0.222$ and trial ($df = 2, 81$; $F = 0.244$; $p = 0.783$) did not significantly influence level of response to the xenic mouse while day ($df = 4, 81$; $F = 4.442$; $p = 0.002$) did. Comparison of fly response to the xenic mouse versus the control revealed that gravid females preferred the xenic mouse early and late in the decomposition process on days 2 (~14%; $df = 1,10$, $F = 16.04$, $p = 0.002$) and 8 (~46%; $df = 1,10$, $F = 21.08$, $p < 0.001$) (Figure 3.11C). Non-gravid females preferred the xenic mouse early in the decomposition process on day 0 (~42%; $df = 1,10$, $F = 17.03$, $p = 0.002$) but switched preferences to the control later in the decomposition process on day 6 (~27%; $df = 1,10$, $F = 5.654$, $p = 0.038$)

(Figure 3.11D). Males had no clear pattern of preference except for day 8 where there was a significant response (~17%; df = 1,10, F = 6.31, p = 0.0308) to the xenic mouse over the control (Figure 3.11B).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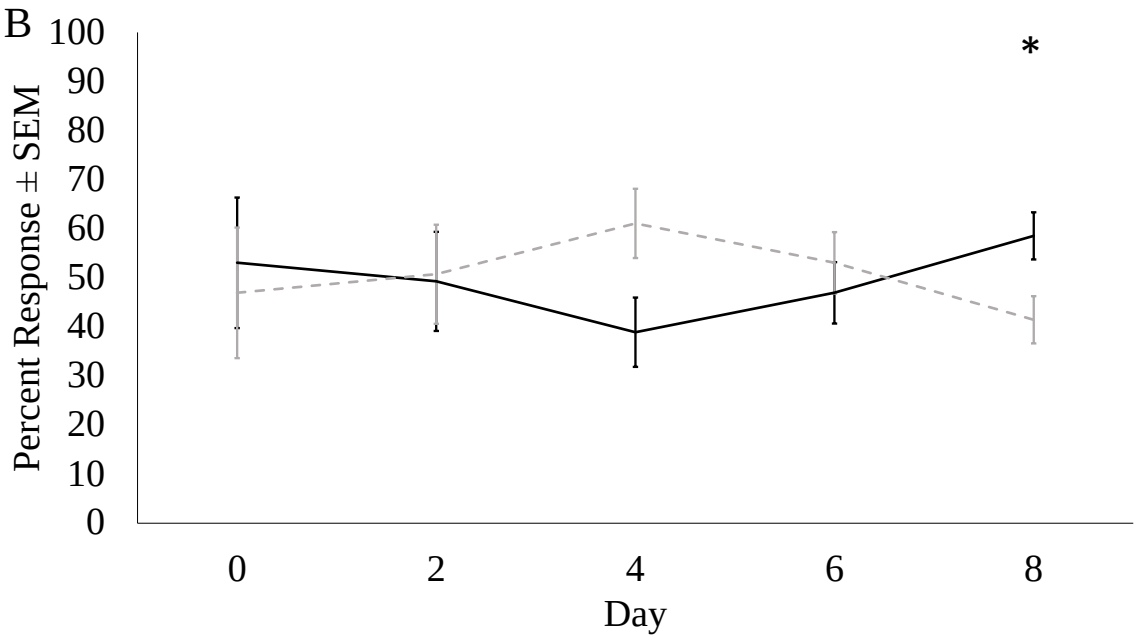
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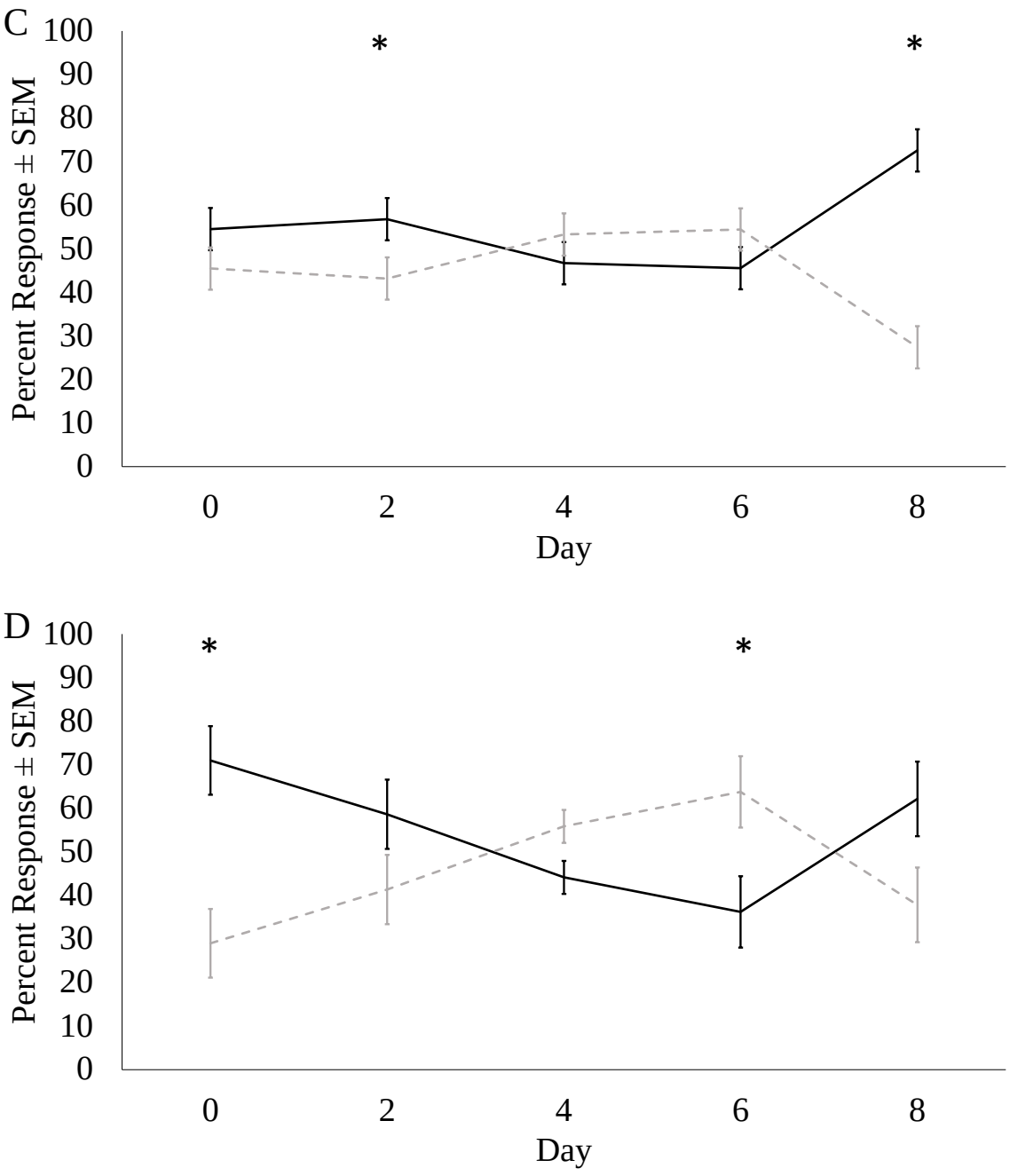


Figure 3.11. Average percent ± SEM response of ~200 7-9 d old *C. macellaria* in an olfaction cube maintained at approximately 21°C to a xenic mouse treatment and control (empty jar) for (A) pooled sex, (B) males, (C) gravid females, and (D) non-gravid females in 8-hour trials; [1]* indicates significance of $p < 0.05$.

8 hour – Axenic v Xenic Total Response – Analysis of total fly response based on pooled sex and physiological state to the axenic moue (treatment) as well as the xenic mouse (treatment) rather than remain in the cube had a significant ($df = 8,15$, $F = 5.13$, $p = 0.003$) trial*day interaction. There was no discernable pattern of order of response level to either treatment combination or not to respond. Overall response ranged from ~22% to ~46% throughout the experiment (Figure 3.12A) with an overall increase in level of response over time.

When analyzed by sex and physiological state, a trial*day*sex interaction was not significant ($p > 0.05$) and was removed from the analysis. Subsequent analysis reveals day ($df = 4,61$, $F = 18.941$, $p < 0.001$), trial ($df = 2,61$, $F = 17.280$, $p < 0.001$), day*trial ($df = 8,61$, $F = 7.947$, $p < 0.001$), and trial*sex ($df = 4,61$, $F = 3.051$, $p = 0.023$) significantly influenced level of response while sex ($df = 2,61$, $F = 0.348$, $p = 0.706$), and day*sex ($df = 8,61$, $F = 1.402$, $p = 0.213$) did not. In general, females responded more early in the decomposition process (days 0-4) and males responded more later in decomposition (days 6-8) (Figure 3.12B).

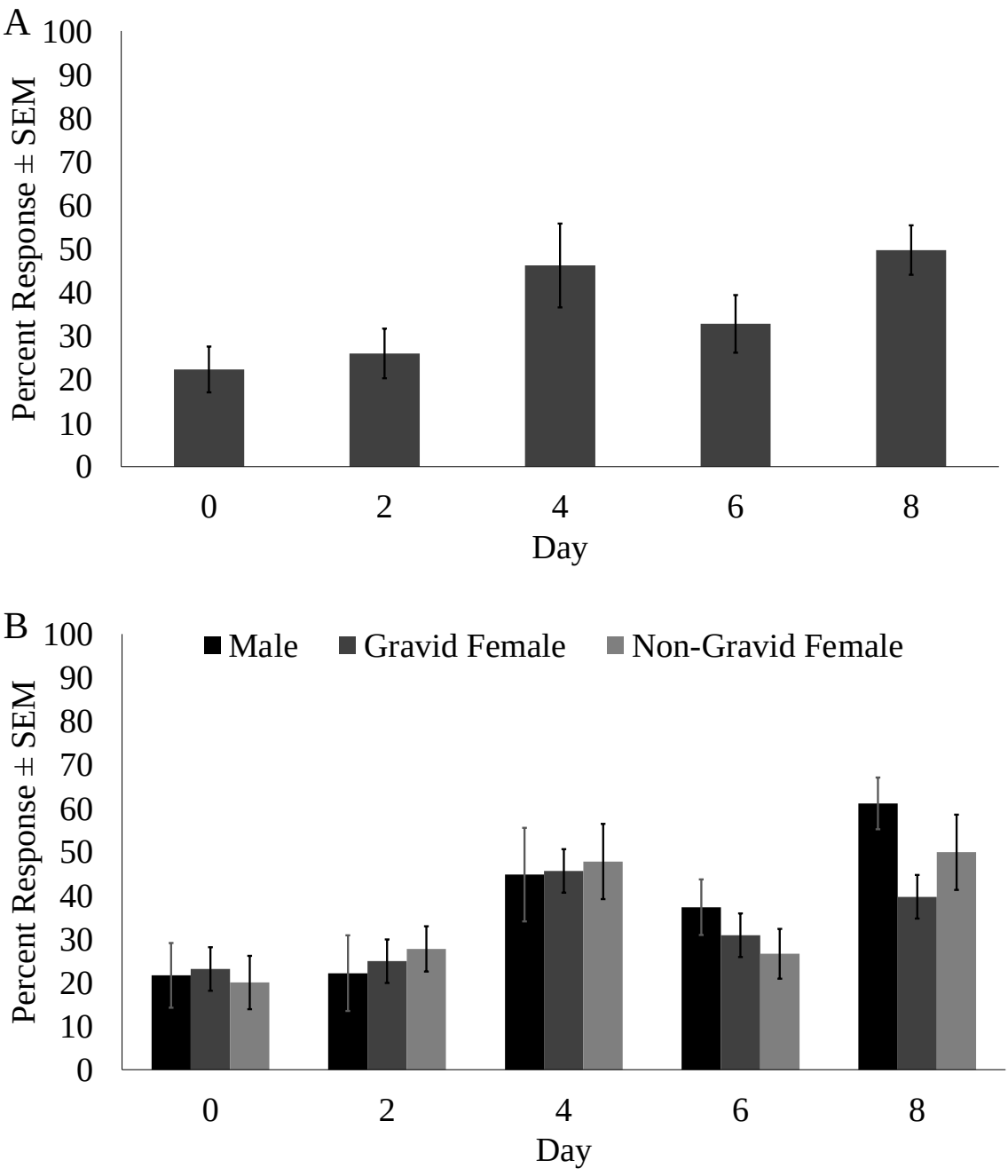
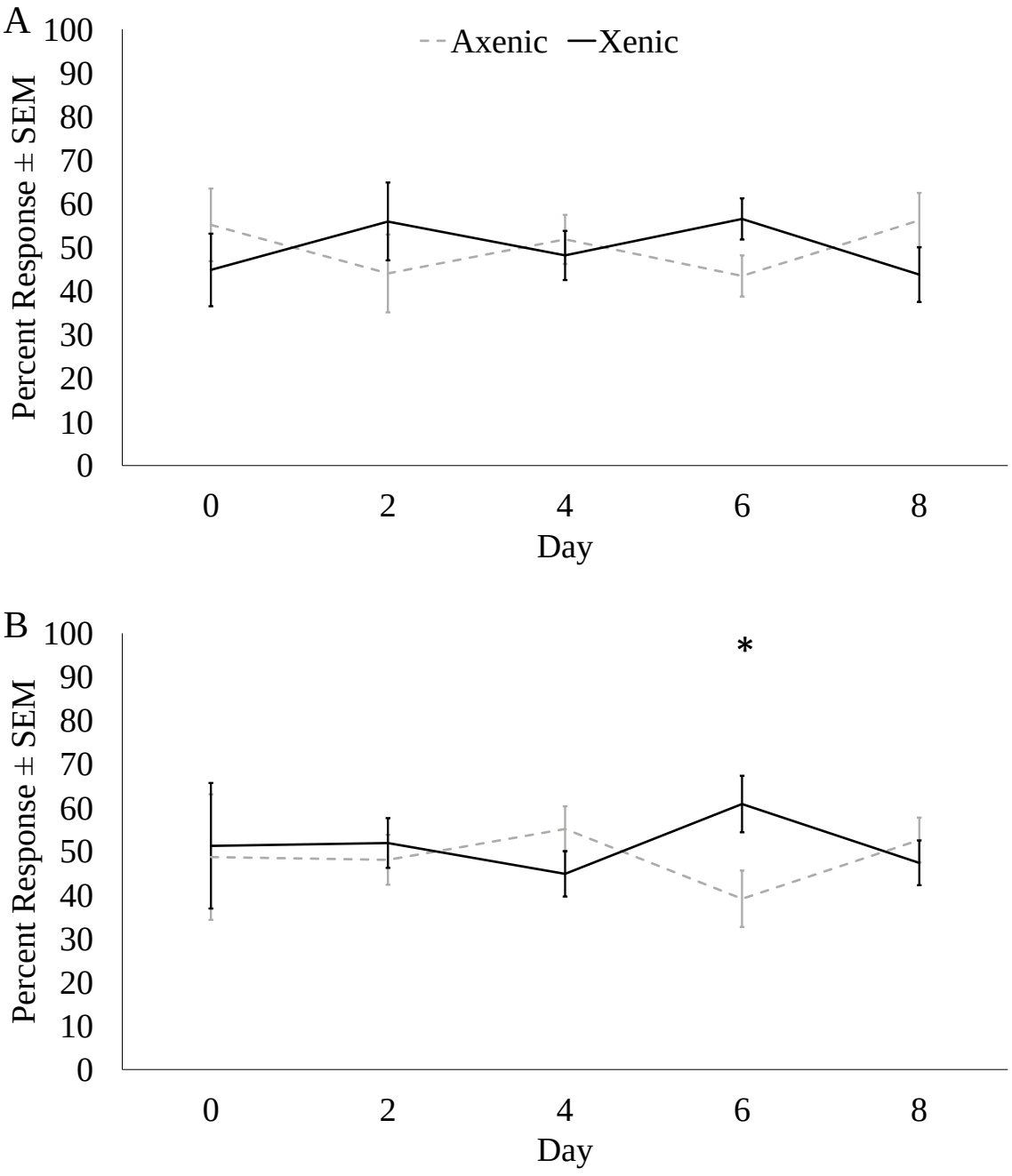


Figure 3.12. Average percent ± SEM response of ~200 7-9 d old male, gravid, and non-gravid *C. macellaria* in an olfaction cube maintained at approximately 21°C to xenic mouse and axenic mouse treatments for (A) overall response with a pooled sex and (B) overall response by sex in 8-hour trials.

8 hour – Axenic v Xenic Treatment Response – No significant interactions ($p > 0.05$) for fly response based on pooled sex and physiological state to the axenic mouse (treatment) as opposed to the xenic mouse (treatment) were discovered and therefore were removed from the analysis. Subsequent analysis demonstrated the level of fly response based on pooled sex and physiological state to the axenic mouse did not significantly differ across trial ($df = 2,23$, $F = 0.021$, $p = 0.979$) or day ($df = 4,23$, $F = 0.697$, $p = 0.602$) (Figure 3.13A). In totality, flies had no clear preference to either treatment throughout the duration of the experiment.

When treatment choice was analyzed by sex and physiological state, fly response to the axenic mouse was not significantly ($p > 0.05$) impacted by a trial*day*sex interaction and was removed. Subsequent analysis demonstrated the level of fly response to the axenic mouse was not impacted by trial ($df = 2,80$, $F = 0.073$, $p = 0.930$), sex ($df = 2,80$, $F = 0.321$, $p = 0.727$), or day ($df = 4,80$, $F = 1.697$, $p = 0.159$). Comparison of fly response to the axenic mouse versus the xenic mouse revealed that males preferred the xenic mouse later in the decomposition process on day 6 (~21%; $df = 1,10$, $F = 5.624$, $p = 0.039$) (Figure 3.13B). Non-gravid females preferred the xenic mouse later in the decomposition process on day 6 (~27%; $df = 1,10$, $F = 8.086$, $p = 0.017$) but then preferred the axenic mouse late in the decomposition process on day 8 (~24%; $df = 1,10$, $F = 5.947$, $p = 0.034$) (Figure 3.13D). Gravid females had no preference between the axenic mouse or the xenic mouse for the duration of the experiment (Figure 3.13C).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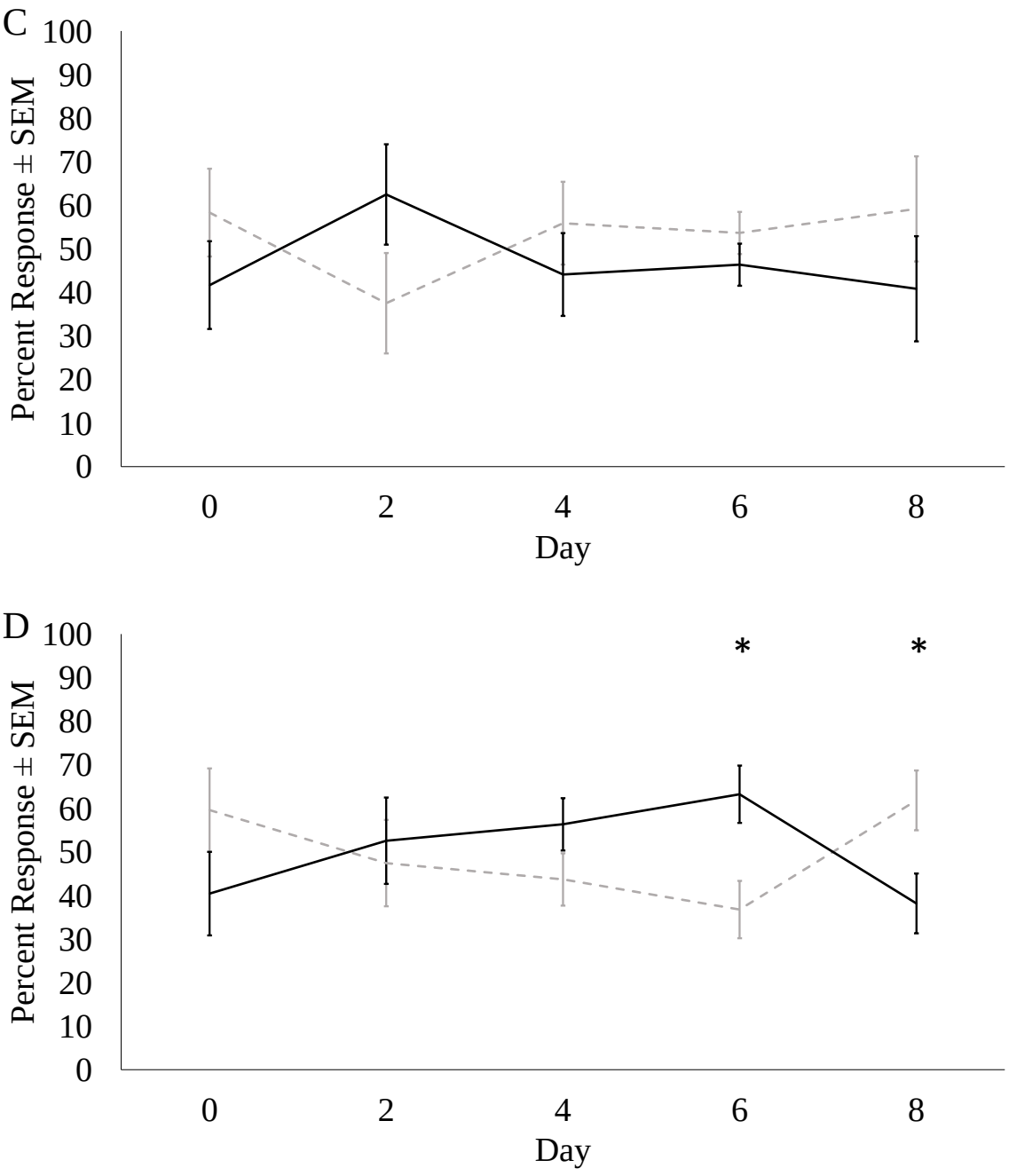

Figure 3.13. Average percent ± SEM response of ~200 7-9 d old *C. macellaria* in an olfaction cube maintained at approximately 21°C to axenic mouse and xenic mouse treatments for (A) pooled sex, (B) males, (C) gravid females, and (D) non-gravid females in 8-hour trials; [1]* indicates significance of $p < 0.05$.

Microbiome Impact on VOC Profiles

GC-MS was used to identify 79 compounds indicating differences between xenic mice, axenic mice, and a control. Volatile profiles of treatments were significantly different from one another (df = 2,350, F = 7.447, p = 0.001) (Table 3.1) and a two-dimensional NMDS ordination analysis explained 97.6% of the variation (stress = 0.1868) in VOC profiles across treatments. There was also a significant difference in volatile profiles across days (df = 1,350, F = 15.574, p = 0.005) (Table 3.1) and an interaction between treatment and days (df = 2,350, F = 2.483, p = 0.001).

Treatment	DF	SS	F	R^2	p value	Adjusted p value
Control v Axenic	1	0.33	2.186	0.009	0.049	0.147
Control v Xenic	1	1.924	11.845	0.047	0.001	0.003*
Axenic v Xenic	1	1.082	6.885	0.028	0.001	0.003*
Day	DF	SS	F	R^2	p value	Adjusted p value
0 v 2	1	0.746	5.695	0.039	0.001	0.01*
0 v 4	1	0.938	6.613	0.044	0.001	0.01*
0 v 6	1	1.405	9.172	0.061	0.001	0.01*
0 v 8	1	2.011	13.727	0.088	0.001	0.01*
2 v 4	1	0.272	1.837	0.012	0.105	1
2 v 6	1	0.558	3.503	0.024	0.011	0.11
2 v 8	1	1.251	8.200	0.054	0.001	0.01*
4 v 6	1	0.372	2.186	0.015	0.046	0.46
4 v 8	1	0.896	5.491	0.037	0.001	0.01*
6 v 8	1	0.422	2.415	0.016	0.027	0.27

Table 3.1. PERMANOVA results testing different comparisons in volatile profiles across a control and two treatments: xenic mouse and axenic mouse as well as across time. Significant differences ($p < 0.05$, Bonferroni corrections applied for multiple comparisons) are indicated by an asterisk (*).

Indicator Compounds

Of the 79 compounds included in the analysis, 9 compounds (11.4%) were indicative of both treatments (axenic mouse and xenic mouse) (Table 3.2) while 7 compounds (8.8%) were indicative of the xenic mouse only (Table 3.3) (Figure 3.14).

Compound	Indicator Value	p value
3-methyl-1-butanol	0.865	0.005
2-pentyl-furan	0.773	0.005
alpha-Methylstyrene	0.713	0.005
Benzaldehyde	0.694	0.005
D-Limonene	0.621	0.005
2-methoxy-furan	0.556	0.005
2,5-Cyclohexadiene-1,4-dione, 2,6-bis(1,1-dimethylethyl)-	0.534	0.005
E-beta-Farnesene	0.498	0.005
Octanal	0.359	0.010

Table 3.2. Indicator compounds associated with both mouse treatments (axenic mouse and xenic mouse carcasses) as compared to the control.

Compound	Indicator Value	p value
Acetoin	0.658	0.005
1-Pentanol	0.62	0.005
Dimethyl trisulfide	0.602	0.005
Ethyl ester butanoic acid	0.487	0.005
1-Hexanol	0.375	0.005
Hexanal	0.244	0.03
1-methyl-2-Pyrrolidone	0.226	0.015

Table 3.3. Indicator compounds associated with xenic mouse carcasses only as compared to the axenic mouse treatment and the control.

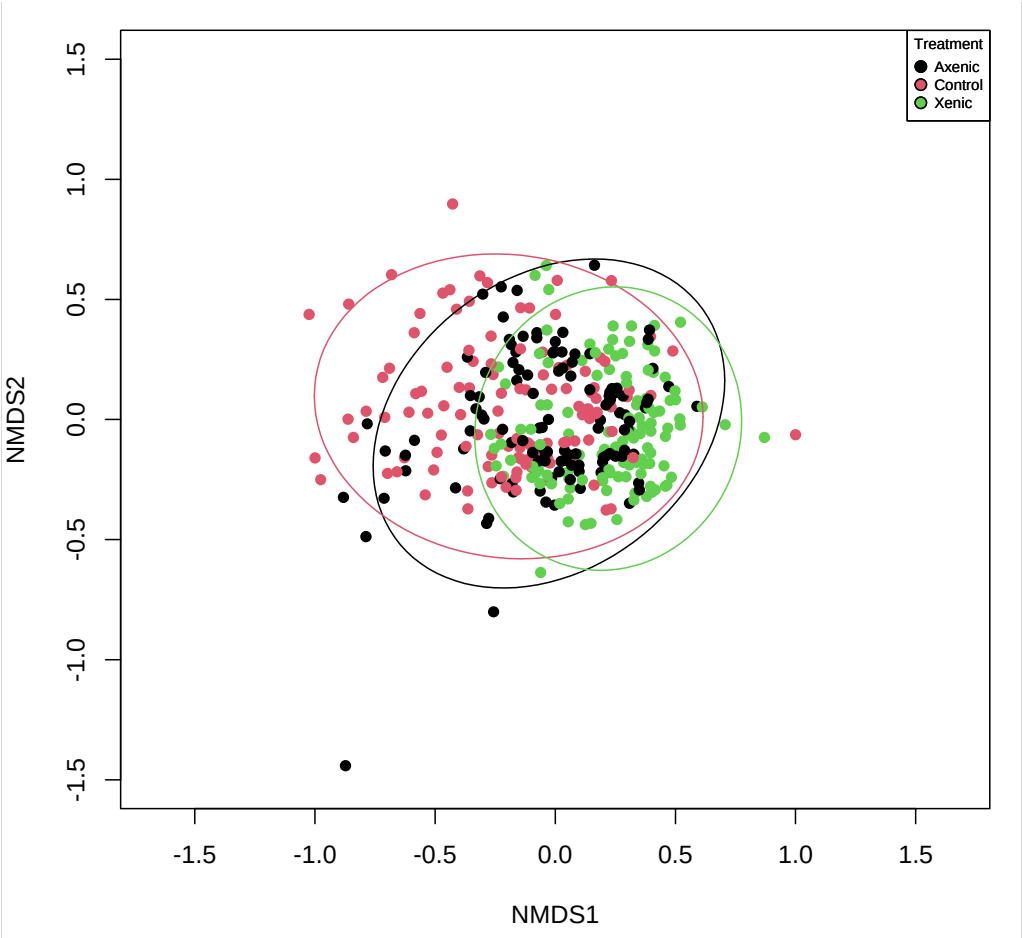

Figure 3.14. Axenic mouse, xenic mouse, and control VOC profiles visualized using a non-metric multidimensional scaling ordination with significantly different communities (df = 2,350, F = 7.447, p = 0.001). This ordination explained 97.6% of the variation (stress = 0.1868) in VOC profiles across treatments. The ellipses represent a 95% confidence interval around the centroid.

VOC Compound Richness

There was an overall increase in the number of compounds produced by both treatments (axenic mouse and xenic mouse) and found in the control over the 8-day period (Figure 3.15). On average, 3.13 more compounds were found on day 8 in the control as compared to day 0, 4.73 in the axenic mouse treatment, and 6.17 in the xenic mouse treatment.

During each collection time point, the xenic mouse produced the greatest number of compounds, followed by the axenic mouse (5.86 – 7.63 compounds less than the xenic mouse), and the least number of compounds were found in the control (3.10 – 6.13 compounds less than the axenic mouse) (Figure 3.15). Both day (df = 4,341, F = 8.132, p < 0.001) and treatment (df = 2,341, F = 88.152, p < 0.001) had a significant effect on compound richness while there was no day*treatment interaction effect (df = 8,341, F = 0.406, p = 0.917) on compound richness.

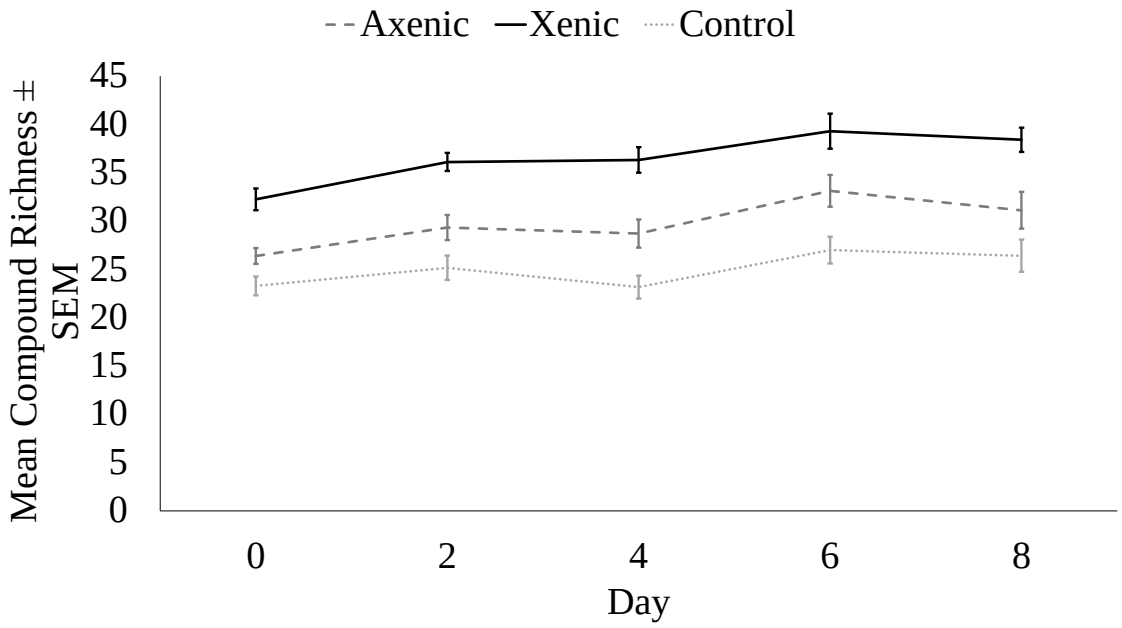

Figure 3.15. Mean compound richness ± SEM of volatile organic compounds from two treatments (axenic mouse and xenic mouse) and a control over an 8-day period.

VOC Compound Evenness

An overall increase in the evenness of VOCs produced by both treatments (axenic mouse and xenic mouse) over the 8-day period (Figure 3.16) was determined. On average, species evenness increased by 0.02 from day 0 to day 8 in the control, 0.04 in the axenic mouse

treatment, and 0.02 in the xenic mouse treatment. During each collection time point, the xenic mouse had the highest compound evenness, followed by the axenic mouse (0.02 – 0.05 less than the xenic mouse), and the least number of compounds were found in the control except on day 4 where evenness was the same between the control and axenic mouse (0.00 – 0.02 less than the axenic mouse) (Figure 3.16). Both day (df = 4,341, F = 3.528, p = 0.007) and treatment (df = 2,341, F = 14.582, p > 0.001) had a significant effect on compound evenness while there was no day*treatment interaction effect (df = 8,341, F = 0.345, p = 0.948).

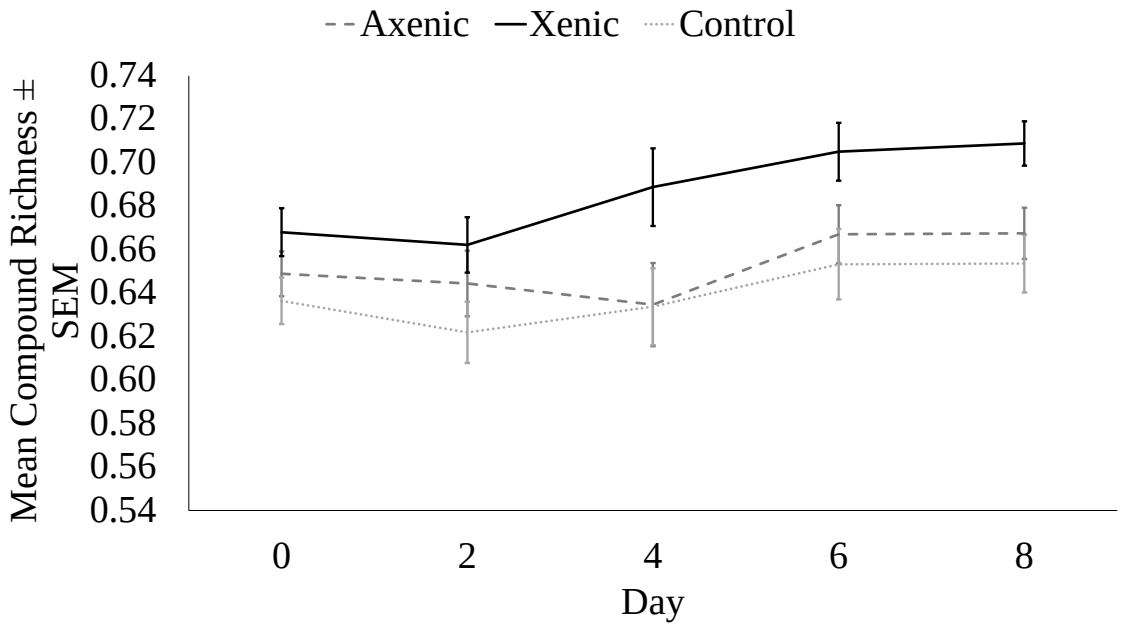

Figure 3.16. Mean compound evenness ± SEM of volatile organic compounds from two treatments (axenic mouse and xenic mouse) and a control over an 8-day period.

VOC Compound Diversity

Using the Shannon-Wiener diversity index (H), an overall increase was detected in the diversity of compounds produced by both treatments (axenic mouse and xenic mouse) and

found in the control over the 8-day period (Figure 3.17). The control diversity increased by 0.12, axenic mouse by 0.14, and xenic moue by 0.27 over the 8-day study period. During each collection time point, the xenic mouse had the highest diversity of compounds, followed by the axenic mouse (0.19 – 0.34 below xenic mouse), and the least number of compounds were found in the control (0.12 – 0.20 below axenic mouse) (Figure 3.17). The diversity of compounds was significantly influenced by day (df = 4,341, F = 6.405, $p < 0.001$) and treatment (df = 2,341, F = 60.308, $p < 0.001$), but not by a day by treatment interaction (df = 8,341, F = 0.470, p = 0.877).

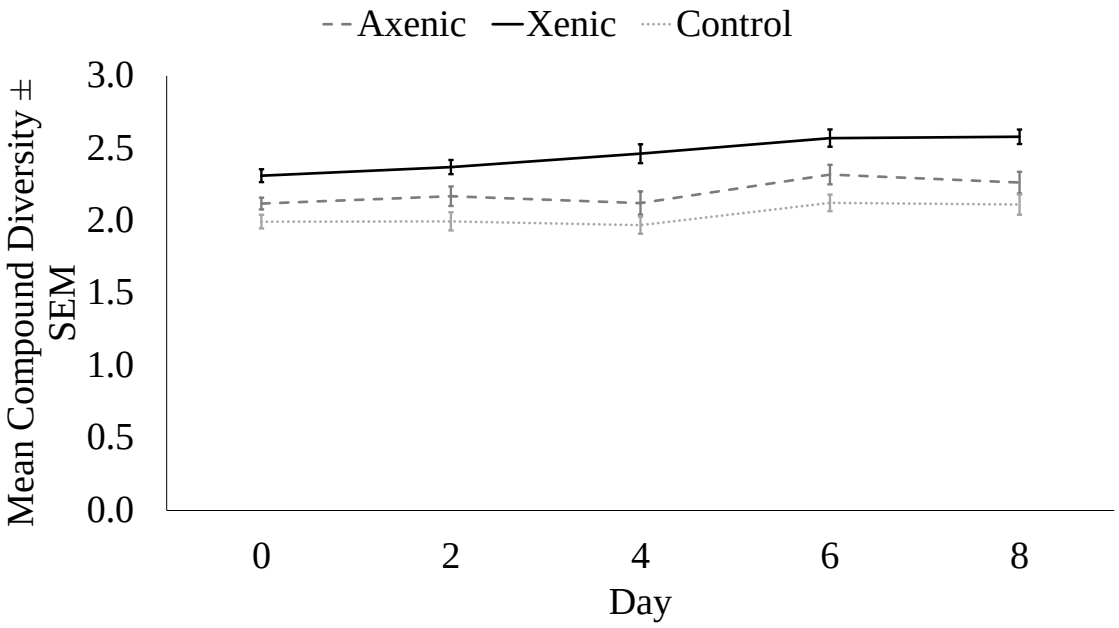

Figure 3.17. Mean compound diversity ± SEM of volatile organic compounds from two treatments (axenic mouse and xenic mouse) and a control over an 8-day period.

Microbiome Impact on Oviposition

When adult flies were allowed direct access to the treatments (axenic mouse carcass, xenic mouse carcass, and control), gravid females laid an average of 0.086g of

eggs on the axenic mouse carcass 0.782g of eggs on the xenic mouse carcass, and no eggs on the control. The absence of microbes resulted in a significant decrease in the mass of eggs laid ($df = 2,15$, $F = 15.49$, $p < 0.001$) (~90%) (Figure 3.18).

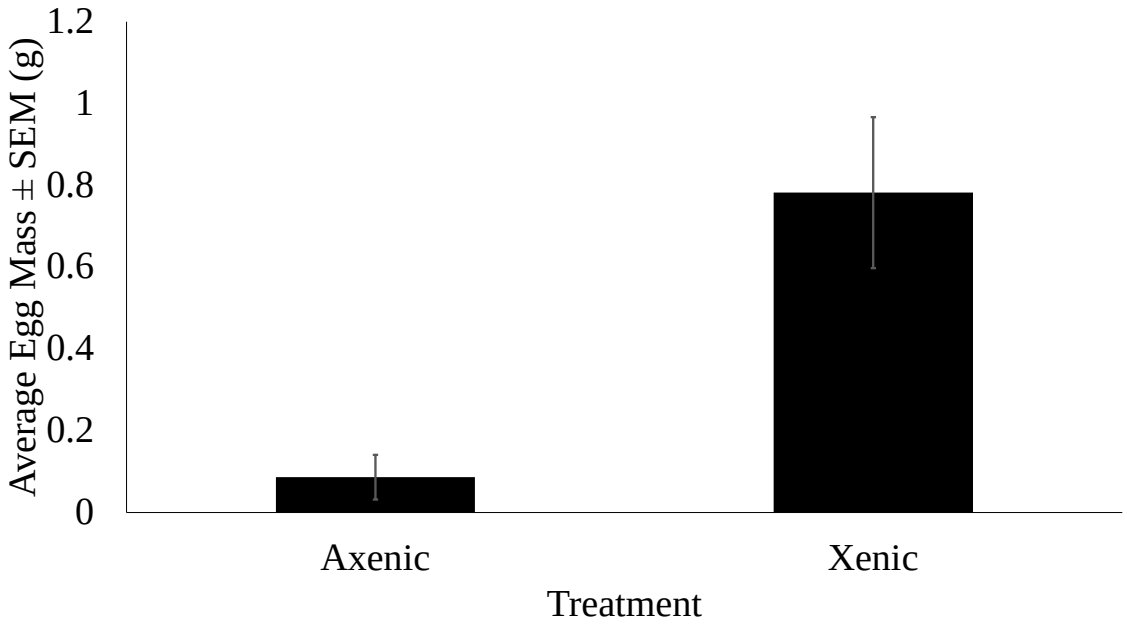

Figure 3.18. Average mass ± SEM of eggs laid on each treatment over a 24 h period by gravid adult *C. macellaria*.

Discussion

Data from this study definitively demonstrate microbes associated with carrion play a crucial role regulating the attraction and colonization by blow flies. Overall, the absence of microbes resulted in 15% less fly attraction; however, if examined 4 days after initiating the experiment, attraction to the xenic mouse was twice as high as the attraction to the axenic mouse. The same can be said with colonization patterns, with 90% less eggs deposited on the aged axenic carrion versus the aged xenic carrion (Figure 3.18). Such

differences could explain variation in attraction and colonization of humans as related to the pre-CI. Exploring the specific microbes regulating such processes could be used to refine PMI estimates in conjunction with entomological data as related to the pre- and post-colonization intervals.

Response in total (i.e., to either treatment) of the flies was dependent on their phenotype (e.g., male, gravid or non-gravid female). For example, males had the greatest response rate (~21%), while gravid and non-gravid females only responded ~15% percent (Figure 6B). However, when dissecting actual responses to treatments, females (gravid and non-gravid) were more intentional showing clear treatment choices (~20% selecting xenic over axenic in comparison to males [~13%]) (Figure 7B-D). These results are not unexpected as sex and physiological state are known to regulate fly response to a resource (Tomberlin et al. 2012). Males are often seeking mates and their temporal attraction to carrion would be similar to non-gravid females (Shorey et al. 1969, Archer and Elgar 2003). In contrast, gravid females could be searching for a suitable oviposition site, while non-gravid females could be searching for a protein resource to stimulate oogenesis as they are attracted to decomposed carrion (Mohr and Tomberlin 2014, 2015b).

Blow fly response to the treatments was temporally dependent. During the xenic mouse and control choices (Figure 3.11), all sexes exhibited a preference for the xenic mouse early in the trial (days 0 and 2), then the control on days 4 and 6, followed by a shift back to the xenic mouse on day 8. This trend could be indicative of the microbial abundance on the resource where day 0 and 2 would be reflective of an “average” microbial abundance and community assembly on a carrion resource and as

decomposition progresses, the microbes are reproducing enough to the point where the resource becomes unattractive or even repulsive to the adults. Later in decomposition on day 8, all three sexes preferred the xenic mouse again, indicating that adults could still utilize the resource. Similar results were recorded for vertebrate carrion in the lab as well as the field. Adult *C. macellaria* preferred uncolonized aged (5 day old) carrion over uncolonized fresh (24 hours old) carrion when given a choice in a lab experiment (Kotzé and Tomberlin 2020). In a field validation study, adult female *C. macellaria* who had undeveloped oocytes were more abundant on carrion approximately 5 days into decomposition when compared to fresh carrion (Mohr and Tomberlin 2015b). The attraction of non-gravid females to more decomposed remains rather than fresh could be the result of a shift in the microbiome that could indicate a reduction in adult fly and microbial competition due to increased larval fly presence and antimicrobial activity.

The VOC profiles produced by each treatment were significantly different from one another. This difference was determined to be driven by seven indicator compounds (Table 3.3). All of these compounds have a bacterial origin (Schulz and Dickschat 2007). For example, one indicator compound for the xenic mouse treatment was dimethyl trisulfide (DMTS), a known blow fly attractant (Chaudhury et al. 2014, Chaudhury et al. 2015). DMTS is produced by the breakdown of the essential amino acid, methionine, which is a sulfur-based compound. *Proteus mirabilis* has been shown to produce DMTS (Tomberlin et al. 2012), is attractive to adult blow flies as a culture (Chaudhury et al. 2016), and is found in the human microbiome (O'Hara et al. 2000)and digestive tract of larval blow flies (Ma et al. 2012). The presence of *P. mirabilis* in the digestive tract is

likely the reason why larval blow flies prefer diets lacking methionine over diets lacking other essential amino acids (Rhinesmith-Carranza et al. 2018) due to the fact that *P. mirabilis* can synthesize methionine (Grabow and Smit 1967). During the oviposition assay after day 8 of the behavior assay, the absence of microbes almost completely inhibited *C. macellaria* oviposition (~90% reduction) indicating that microbes are an important indicator when evaluating resource choice for oviposition by a gravid female. This aligns with results from other studies which show microbes (DeVaney et al. 1973, Chaudhury et al. 2010) and mVOCs (Chaudhury et al. 2016) increase the attractiveness of substrates to gravid female blow flies.

Practitioners in forensic microbiology have utilized the microbiome composition and postmortem microbial succession to determine an estimate of the PMI (Benbow et al. 2015, Johnson et al. 2016, Metcalf et al. 2017), with succession studies excluding insect access (Metcalf et al. 2013), including insect access (Pechal et al. 2013a, Pechal et al. 2013b), and even delaying insect access (Pechal et al. 2014). The current study expands on this knowledge by including a VOC profile reflective of the microbial succession occurring within carrion which could link the fields of forensic microbiology, forensic chemistry, and forensic entomology. Future research in this discipline should inoculate axenic mice with single microbes known to be in the human microbiome to determine the effect on VOC profiles, and potentially expand to combinations of microbes.

Microbial VOCs clearly govern insect attraction through VOCs in an animal or carrion system (DeVaney et al. 1973, Ma et al. 2012, Tomberlin et al. 2012, Liu et al. 2016b, Tomberlin et al. 2017) as well as in bacteria-mediated plant-insect interactions

(Bartelt and Wicklow 1999, Sugio et al. 2015) for various purposes. Such conclusions should not come as a surprise given the relationship between microbes and insects associated with carrion. However, the relationships between these actors governing decomposition are complex. In some instances, microbes are pathogenic to these insects (Wright et al. 2004), while in other instances they are beneficial (Grabow and Smit 1967, Tomberlin et al. 2017). The same can be said from the microbial perspective where blow fly activity on decomposing remains can be detrimental (Pöppel et al. 2015) or beneficial, such as a transportation vector (Junqueira et al. 2017).

If microbes are truly the driving factor in blow fly attraction to a carrion resource, changes in the carrion microbiome could alter blow fly attraction by either increasing or deterring attraction. Kotzé and Tomberlin (Kotzé and Tomberlin 2020) observed such variations in colonization patterns of vertebrate remains; however an assessment of microbial communities in conjunction with this observation was not made. Future studies should assess blow fly attraction and colonization preferences in addition to microbial abundances during each time point.

Shifts in the microbial community associated with vertebrate remains (i.e., elimination as in the case of the current study) impacts the duration of the pre-CI as related to insect attraction and colonization (Tomberlin et al. 2011). Unfortunately, an in-depth understanding of this interaction is not knot known. The recognized limitation to the application of forensic entomology is the inability to account for the duration of the pre-colonization interval as part of the overall PMI which violates at least one assumption

associated with using the term such as immediate oviposition after death (Tarone and Sanford 2017).

Microbiome research such as the current study could provide a hint as to how the microbiome of the host regulates attraction of primary colonizers. These studies could include a complete known microbiome or could begin with one known microbe found in carrion and determine individual effects on VOC production and insect attraction. Understanding how microbes affect insect attraction and colonization can be a critical addition to the field of forensic entomology.

This study determined that microbes are the influence behind attraction and colonization of adult *C. macellaria* to a carrion resource. Future work should expand on other species of primary colonizers to ensure this is not an anomaly solely for this study species and use other types of ephemeral resources such as fruit or manure in which dipterans utilize for reproductive and nutritional purposes. Although domesticated pigs are the generally accepted human analogue in forensic science studies (Matuszewski et al. 2019), future studies comparable to the current study should continue using the mouse model as it allows for easy manipulation of the host microbiome. Manipulation of the mouse microbiome is already an established practice, and decomposition ecology could establish a mutually beneficial relationship between the two fields as culled colony mice could still serve as experiment models rather than being discarded.

While these results are revealing, life history of the decedent is important. Overall health has been shown to impact an individual's microbiome, and these health conditions can still be detected during post-mortem microbial biodiversity sampling, specifically

Rothia sp. were in higher abundance in the mouth of individuals with heart disease (Pechal et al. 2018). Research stemming from this study should explore the effect of host nutrition on the microbiome and subsequent primary colonizer attraction and colonization as well as the VOC profile produced. For example, mice fed with a sulfur amino acid restriction diet (0.12% methionine) versus a control (0.86% methionine) had higher abundances of Firmicutes, Clostridiaceae, and Turicibacteraceae bacteria and lower abundances of Verrucomicrobia bacteria than the control showing that diet has a significant influence on the gut microbiome of the host (Nichenametla et al. 2020). Another field where this work could be applicable is expanding on the known impact different medical treatments (such as antibiotic treatment or chemotherapy (Shuwen et al. 2020)) have on the microbiome and how those alterations could impact VOC profiles and subsequent insect colonization if that patient were to parish. Acute or chronic drug use has also been shown to alter the host microbiome which could impact VOC profiles and subsequent insect colonization if that individual were to parish. For example, chronic alcohol consumption was demonstrated to decrease the abundance of Bacteroidetes and increase the abundance of Proteobacteria in the colonic microbiome of study subjects (Mutlu et al. 2012). This study provides the foundation for decomposition ecologists and other fields of study to utilize the variation in microbiome and expand on the current knowledge of multi-trophic interactions involving microbes.

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4. CONCLUSIONS

I conducted a series of experiments that investigated the role microbes play in the attraction to and colonization of mouse carrion by adults of a primary carrion colonizer species, *Cochliomyia macellaria* (Fabricius) (Diptera: Calliphoridae), and investigated how fly sex (e.g., male, gravid female, non-gravid female) affected their responses. I also examined the impact of excluding microbes on carrion on its volatile organic compound profile as relates to the behavioral responses of *C. macellaria*. I also determined and implemented optimal parameters for using a dual-choice cube olfactometer to explore adult *C. macellaria* behavior.

In the first study (Chapter 2) (Flint and Tomberlin 2020), I conducted a series of experiments to optimize the response of adult *C. macellaria* to treatments in a dual-choice cube olfactometer. For this experiment, I measured the impacts of fly adjustment time (0, 15, 30, 45, and 60 min), experiment duration (2, 4, 6, and 8 hrs), presence or absence of sugar and water, and insect netting type (new or re-used) on overall response (i.e., did they select a treatment) and treatment choice by *C. macellaria* adults. Adult sex (male, gravid and non-gravid female) was also analyzed to determine the impact on fly response and preference.

I determined that ~70% of all sexes responded to the liver with the 30-min adjustment period compared to ~50% in all other adjustment period lengths. The highest overall response levels were exhibited during 8-hour trials (~35% increase), but significant response to treatments diminished as trial length increased. Gravid and non-gravid female

response decreased by ~35% when sugar and water were present in the cube and reuse of insect netting had no impact on fly response. From these studies, we can conclude that *C. macellaria* preference for olfactory stimuli can be suitably assessed following the guidelines outlined in this chapter.

One limitation of this study is the design of the cube olfactometer only including two choices, but the design could be modified to include up to 4 choices if desired for future studies (Yu et al. 2013). This olfactometer is a viable alternate to the Y-tube olfactometer (Urech et al. 1994) for flying insects. Future work should expand on these findings by determining optimal parameters for other species of blow flies that are commonly used in behavioral assays due to biological and behavioral differences between species For example, *Lucilia sericata* (Meigen) (Diptera: Calliphoridae) provided significant results after 24 h trials using the dual-choice cube olfactometer in previous studies (Ma et al. 2012, Tomberlin et al. 2012). This difference in behavior could potentially be explained by biological differences, where *C. macellaria* prefers warmer weather compared to *L. sericata* (Hall 1948). Warmer weather will increase the rate of decomposition of carrion (Benbow et al. 2013) and alter VOC profiles, creating a smaller temporal window of suitability for oviposition.

My second (microbiome impact on attraction), third (sex and ovarian development impact on attraction), fourth (microbiome impact on volatile organic compounds), and fifth (microbiome impact on oviposition) objectives were all combined into one chapter (Chapter 3). This chapter used xenic (specific-pathogen free) and axenic (germ-free) mice as a carrion resource to investigate the impact microbes have on entomologically relevant

stages of the vertebrate decomposition process such as detection, location, and colonization of a carrion resource (Tomberlin et al. 2011).

The absence of microbes within a carrion resource significantly (~15%) reduced adult *C. macellaria* attraction overall. Sex and physiological state (undeveloped or developed ovaries) had a significant impact on attraction to xenic and axenic mice, with males responding differently than gravid and non-gravid females. Males (~21%) responded more than gravid (~15%) and non-gravid females (~15%), but females showed a more intentional response to the xenic mouse over the axenic mouse (~20%) compared to males (~13%). Xenic and axenic mice exhibited significantly different volatile organic compound (VOC) profiles, with seven compounds specific to xenic mice, most of which have a microbial origin such as dimethyl trisulfide. The absence of microbes within a carrion resource also significantly (~90%) reduced adult *Cochliomyia macellaria* oviposition.

One potential limitation to this study is that the oviposition was completed only on day 8 of the experiment. Ideally, oviposition would be examined throughout decomposition via replication of the mouse treatments and repeated oviposition assays over time as shown in Pitts and Wall (Pitts and Wall 2004) to determine how the change in microbiome over time affects oviposition. Field and laboratory research show that oviposition preferences of blow flies follow a general trend, dependent on decompositional progress of the carrion resource with preferences of early or later decomposition (Anderson and VanLaerhoven 1996, Erzinçlioğlu 1996, Kotzé and Tomberlin 2020). Future studies should explore the microbiome further, perhaps by

adding one microbial species at a time and determining the effect those variations have on behavior, VOC production, and oviposition. A microbe of immediate interest is *Proteus mirabilis* due to its natural presence in the gastrointestinal tract of humans (Drzewiecka 2016) and blow flies (Ma et al. 2012), attractiveness to blow fly adults seeking an oviposition site (Chaudhury et al. 2016) through VOC products (dimethyl trisulfide) (Tomberlin et al. 2012, Chaudhury et al. 2015), and its ability to produce the essential amino acid methionine (Grabow and Smit 1967). The studies cited in the previous sentence have been conducted in isolation or using pure *P. mirabilis* so inoculating a mouse with *P. mirabilis* before euthanasia to test VOC production and blow fly attraction after death will validate the results in an applied system. Other microbial groups of interest are Clostridia, Bacilli, and Bacteroidia as they have been identified as the most changing bacterial taxa groups after death (Metcalf et al. 2013) and include species such as *Clostridium difficile* and *Bacteroides fragilis*. Through this method, it can be determined which microbial species is/are the most influential on behavior and oviposition of primary colonizers of carrion, therefore allowing an increase in precision of a pre-colonization interval estimate.

My research provides a key clue to a previously unexplored concept in decomposition ecology. In a forensic context, it has been established and generally accepted that there is a period of time between death and insect colonization known as the pre-colonization interval (Tomberlin et al. 2011). Factors affecting insect colonization such as time of day (Zurawski et al. 2014), location of remains (Anderson 2011), and physical barriers such as clothing (Matuszewski et al. 2016) have been studied, but biotic

impacts on insect colonization have previously been neglected in research. Post-mortem microbial succession research has provided valuable information about biotic changes occurring within vertebrate remains (Metcalf et al. 2013, Pechal et al. 2013b, Pechal et al. 2013a, Metcalf et al. 2017) and how microbes affect VOC production, which prompted the hypothesis that microbes have a role in attracting primary colonizers to a carrion resource. This dissertation has established that microbes have a significant impact on VOC production from carrion, insect attraction to a carrion resource, and insect oviposition on a carrion resource. Although this is the first step, it provides a crucial foundation to continue exploring post-mortem microbial changes and how microbiome variations can impact insect colonization of remains. Knowing the impact of the microbiome on insect colonization can allow for a more precise estimate of the pre-colonization interval, which when combined with a post-colonization interval estimate will result in a more accurate and precise post-mortem interval estimate from an entomological perspective.

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