VOLATILE EMISSIONS FROM CARRION AND THEIR EFFECT ON ATTRACTION AND OVIPOSITION BEHAVIOR OF *COCHLIOMYIA MACELLARIA* FABRICIUS (DIPTERA: CALLIPHORIDAE) AND *HERMETIA ILLUCENS* (L.) (DIPTERA: STRATIOMYIDAE)

A Dissertation

by

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ABSTRACT

Insect succession on carrion is highly variable and can be affected by a multitude of biotic and abiotic factors, including, but not limited to, temperature, carcass type, species interactions, genetics, and scavenging. The functional utilization of carrion by various insects has not been extensively investigated, since the majority of carrion associated insects are assumed to be feeding on the carcass, thus investigation into the functional groups present on carrion has not yet been clarified. The study presented here was conducted to evaluate the drivers of oviposition on carrion resources, and clarify the utility of arthropod succession on carrion to estimate the time of colonization (TOC) estimates for insects present at vertebrate remains. The present study aimed at identifying the volatile organic compounds (VOC) emitted from variably aged carrion resources, as well as colonized and uncolonized rat carcasses by inter- and intraspecific species, and evaluating the responses of two carrion-associated flies to these resources. The secondary screwworm, *Cochliomyia macellaria* (specialist), and black soldier fly, *Hermetia illucens* (generalist), were selected for investigation in this study. Both species were exposed to fresh and aged (5 d) rat carcasses, colonized by *C. macellaria* or not, and oviposition responses at each carcass type documented. *H. illucens* was also provided with a grain-based diet (control) for oviposition, to account for their wider dietary breadth. There was a significant difference in the VOC profiles obtained through headspace analysis from the different treatments, with indicator compounds all being of microbial origin. Both species exhibited a significantly greater preference to oviposition on the aged rat carcasses than at fresh carcasses, regardless of colonization status. However, when a grain-based diet was included, *H. illucens* exhibited a significantly higher response to this treatment. The results of this study allowed for a quantification of compounds emitted during vertebrate decomposition under various treatments, and provided insight into the drivers governing arthropod succession in the two species examined. *C. macellaria* can colonize fresh or aged carrion, suggesting general conclusions about this species as solely colonizing carrion soon after death are not always accurate. Furthermore, *H. illucens*, while known to colonize carrion, is potentially responding to VOCs associated with decaying plant matter (i.e., control) rather than those associated with the carrion resource. Successional patterns in carrion-associated Diptera are highly variable, and should be reevaluated for use in TOC estimates for criminal investigations. Caution should also be exercised when assuming that all species associated with carrion are feeding on the carcass to meet their developmental requirements.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1.1 Decomposition processes

The decomposition process of any organic organism is one that proceeds over time, characterized by specific stages, and organisms considered to dominate each stage (Byrd and Castner 2010, Catts and Haskell 1990, Michaud *et al.* 2015, Rivers and Dahlem 2013). During this time, the organism is a resource pulse, providing, amongst other things, food for numerous other organisms, ranging from vertebrates to invertebrates and microbes (Benbow *et al.* 2019, Holt 2008, Yang 2004, Yang *et al.* 2008). A decomposing body is an ephemeral one, which will deplete over time. The time taken to depletion will vary, depending on consumers present and environmental conditions (e.g., wind, rainfall, temperature and exposure to sunlight) (Campobasso *et al.* 2001, Payne 1965). This nutrient-rich resource not only provides a direct source of food for communities, but may also serve to indirectly enrich the environment during the decomposition process by the release of nutrients and minerals into the soil (in the case of a resource being in an outdoor environment), such as phosphorous, sulfur and nitrogen which are essential for faunal succession and microbial growth (Macdonald *et al.* 2014, Parmenter and MacMahon 2009).

Due to the largely patchy and temporary nature of a decomposing organism, this resource often supports only a single generation of multiple species (excluding microbes) that interact with it and one another (Atkinson and Shorrocks 1981, Beaver 1977). The carrion resource promotes species diversity by providing a foundation on which ecological succession can begin (Beasley *et al.* 2012, Philip *et al.* 2013, Towne 2000). Many species utilizing this resource will come into contact with each other at some point during the decomposition process (Benbow *et al.* 2013, Mohr and Tomberlin 2014, Pechal *et al.* 2014). These species interactions can include predation, competition, commensalism, parasitism, mutualism, and symbiosis (Howe and Westley 1990, Janson 1989, Paine 1966, Williams *et al.* 2002). Many arthropods colonizing decomposing matter can be considered ecological indicator species, as they are able to provide information regarding the environmental conditions at the time of colonization, as well as changes in the environment during the time when the resource was colonized, such as physiological changes, or the addition or presence of exogenous chemicals during the colonization and feeding process (Carvalho *et al.* 2000, Niemi and McDonald 2004).

Although the decomposition process occurs in predictable stages (Payne 1965), there are multiple biotic and abiotic factors that can alter the duration of each stage. Biotic factors affecting decomposition include the presence or absence of arthropods (Payne 1965, Pechal *et al.* 2014, Simmons *et al.* 2010), the presence and activity of vertebrate scavengers (DeVault *et al.* 2004), and microbial activity (Carter *et al.* 2015, Jordan and Tomberlin 2017, Lauber *et al.* 2014), while the abiotic factors of importance include temperature (Archer 2004, Shean *et al.* 1993), precipitation (Archer 2004) and solar radiation (Bass 1997). In addition, numerous studies have refuted the validity of describing the decomposition process in stages, and argued that decomposition occurs more as a continuum (Carter *et al.* 2007, Goff 2009, Schoenly and Reid 1987), with overlapping physiological processes. For example, the advanced decay stage is usually characterized by active arthropod feeding, but in the absence of arthropods, there appears to be no active decay stage, with remains changing from bloat to a deflated liquified mass rather than distinct active and advanced decay stages (Bachmann and Simmons 2010).

1.2 Ecological succession

Ecological succession can be defined as the change in community structure on a resource over time (Clements 1916, Gleason 1917). Time scale can be relatively short (e.g., a century, as seen in the ecosystem recovery at Krakatau) (Wilson 1999) or millions of years (e.g., ecosystem recovery after a mass extinction event) (Sahney and Benton 2008). In general, the community begins with a relatively low number of founding species, and over time, develops into an increasingly complex array of species (Baker 1989, Burgess *et al.* 1981, Harris *et al.* 1984). Various mechanisms have been proposed as drivers of ecological succession, including competition, migration, and reaction (Clements 1916). Each of these drivers has specific characteristics and the nature of the ecosystem will define which of these drivers will begin the succession process. There may be a newly emerged ecosystem that is as yet not colonized (e.g., a river that has dried up and leaves behind moist, nutrient-rich soil), whereby multiple species and communities will look to take advantage of the resources available (Golley 1977, Luken 1990). There may also be a large-scale disturbance which makes way for new species to move into the area and colonize an area that was previously colonized by other species (e.g., a wildfire that destroys a large area of forest habitat), utilizing nutrient remnants from the previous communities (Golley 1977, Luken 1990).

Perhaps some of the most well-known examples of ecological succession have been demonstrated in algal communities in intertidal zones, and with plants in terrestrial systems after a disturbance. Farrell (1991), Sousa (1979) and Whalen *et al.* (2016) all studied variation in algal communities in intertidal zones in California, USA. All authors found strong evidence of community structure affected by successional relationships, with factors, such as competition, being the main drivers of successional patterns. These three studies all demonstrated the presence of pioneer and secondary species, both together and in isolation from one another, and showed the effects of overall succession in intertidal zones under these conditions. These studies also provide insights into the mechanisms driving succession, particularly after a disturbance has disrupted the natural balance of the ecosystem. Other studies (Becker *et al.* 2017, de Carvalho *et al.* 2012, Di Palo and Fornara 2017, García de León *et al.* 2016, Szefer *et al.* 2017, Woods 2017) have demonstrated the role and mechanisms of ecological succession in plant communities, showing shifts in community composition with time from hardy, pioneer species to more nutrient-rich species.

1.3 Succession on carrion

Arthropod succession in carrion differs from classical ecological succession in a few key aspects. Firstly, while most patterns of succession are observed over extended periods of time, from decades to centuries (Sahney and Benton 2008), succession on carrion occurs on a significantly shorter time scale, with some studies reporting insect activity in the dry remains stage around 271 days post-exposure (Anderson and VanLaerhoven 1996). Secondly, most classical patterns of succession in forests or intertidal systems show a pattern from pioneer or foundation species, culminating with an apex or climax community (Horn 1974). The community composition of arthropods on carrion, while diverse and complex, do not show a climax or apex community. Due to the ephemeral nature of vertebrate remains, the community changes fairly constantly, and can cease fairly rapidly (Anderson and VanLaerhoven 1996). Lastly, while many forest and intertidal succession systems are fairly large scale, spanning tens of kilometers (Duncan and Duncan 2000), succession on carrion occurs on smaller scales. Even in mass mortality events, carcass distribution rarely spans more than a few kilometers (Fey *et al.* 2015). As with most patterns of ecological succession, communities associated with terrestrial carrion resources arrive and depart in predictable patterns. Because of these predictable patterns, the taxa present on carrion at the time of discovery are often used to make inferences and estimations regarding the time of colonization (TOC), in conjunction with climatic data (Amendt *et al.* 2007). The timing of arrival and departure to a carrion source is often related to the stage of decomposition, a relationship which has been studied extensively under a variety of conditions (Anderson 2000, Campobasso *et al.* 2001, Carvalho *et al.* 2000, Centeno *et al.* 2002, Richards and Goff 1997, Voss *et al.* 2011, Voss *et al.* 2008). It is important to note here that the timing and arrival of arthropods to a carrion resource are not discrete events; often there is an overlap in the species arriving and departing the resource, as well as no clear distinction between the stages of decomposition, making discrete identification of colonization stage and decomposition stage somewhat problematic (Moura *et al.* 2005, Schoenly and Reid 1987).

In cases of carrion colonization, the most widely represented insect order is Diptera (true flies), followed by Coleoptera (Campobasso *et al.* 2001, Centeno *et al.* 2002, Magni *et al.* 2013, Reibe and Madea 2010, Zanetti *et al.* 2014). At the time of exposure, the carrion source is presumed to be in the fresh stage of decomposition, at which time flies of the families Calliphoridae and Muscidae colonize the body (Amendt *et al.* 2004, Centeno *et al.* 2002, Early and Goff 1986, Goff 1993), and generally remain until the dry decay stage. At the onset of the bloat stage, beetles belonging to Histeridae (clown beetles), Silphidae (carrion beetles) and Staphylinidae (rove beetles) are typically present. The beetles feed on blow fly larvae present on the body, as well as on the decomposing tissue (Centeno *et al.* 2002, Early and Goff 1986). In the later (dry) stages of decomposition, beetles from the families Dermestidae and Cleridae are present and feed on the bone remnants, hair, and fungi that remain on the body (Centeno *et al.* 2002, Early and Goff 1986).

The stages of decomposition of remains are broadly defined, and there are no clear distinctions between the different stages (Swann *et al.* 2010). There are, however, defining characteristics that are indicative of the different stages. In light of this, Tomberlin *et al.* (2011) redefined these stages to encompass a broader distinction of stages of decomposition as seen from an entomological perspective, distinguishing stages of death into a pre- and post-colonization interval.

The successional patterns of the insects on carrion also vary widely with season, based on the species, which are active throughout different times of the year. Centeno *et al.* (2002) showed clear differences between colonization patterns during winter and summer in Buenos Aires, Argentina, between exposed and sheltered cadavers, and Benbow *et al.* (2013) found species-specific seasonal colonization differences in Ohio, USA. While the species present are very similar between the seasons, the timing of arrival and departure of the distinguishing families varies greatly. Although these patterns have been mirrored by numerous studies and have been used as known indicators for a reliable estimate of the time of colonization (Battán Horenstein *et al.* 2010, Centeno *et al.* 2002, Grassberger and Frank 2004, Gruner *et al.* 2007, Magni *et al.* 2013, Michaud and Moreau 2009, 2011, 2017, Michaud *et al.* 2015, Richards and Goff 1997, Voss *et al.* 2008, 2011), Michaud *et al.* (2015) noted that successional studies which have investigated plant successional patterns may not accurately apply to carrion successional patterns. Plants, particularly those that colonize an area after a disturbance, tend to be long lived and persistent over periods of time (Bazzaz 1979), whereas insects colonizing carrion tend to remain on the resource for shorter periods, consuming what they need before vacating the area to continue their next stage of life in another location (Schoenly and Reid 1987). Plant assemblages will also overlap in generation and species (Bazzaz 1979, Walker and Wardle 2014), whereas arthropod succession on carrion appears to be more distinguishable both by time and species present (Schoenly and Reid 1987).

Arthropod colonizers of carrion are broadly categorized as either primary or secondary colonizers (Goff *et al.* 1988). Primary colonizers fall within the first sere of arthropods to reach the carcass, sometimes arriving at a resource within minutes after environmental exposure (Byrd and Butler 1996) and include Diptera as well as Coleoptera. Current hypotheses pose that primary colonizers prepare the carcass and trigger many of the physical decomposition processes, such as tissue breakdown (Anderson 2016), as well as playing a vital role in inhibiting microbial growth that could prove detrimental to larval development (Barnes *et al.* 2010). Once a large portion of the biomass has been consumed by the primary colonizers, the second sere of colonizers, known as secondary colonizers, colonizes the resource. Secondary colonizers include Muscidae (i.e., filth flies), Sarcophagidae (i.e., flesh flies), Calliphoridae (i.e., blow flies) and Coleoptera (Schoenly and Reid 1987). Because the process of decomposition consists of multiple stages over time, the arthropods colonizing each stage are most adapted to breaking down and recycling a specific part of the resource, leaving the remains for the next wave of arthropods more suited to breaking them down.

1.4 Variation in feeding strategies

Both vertebrates and invertebrates have developed feeding strategies which vary depending on their habitats and niches. Besides herbivorous, omnivorous, and carnivorous lifestyles, organisms have also developed feeding strategies which are classified as either generalist or specialist strategies. Generalist feeders are able to survive on a wide variety of resources in order to meet nutritional requirements, while specialist feeders have more limited range of foods with which to meet nutritional requirements (Ali and Agrawal 2012, Devictor *et al.* 2008, Hanski *et al.* 1991). Omnivores are often generalist feeders, while herbivores and carnivores are considered specialist feeders (Ali and Agrawal 2012, Devictor *et al.* 2008, Hanski *et al.* 1991).

As with most lifestyle strategies, there are associated advantages and disadvantages. Generalist species may have access to a wider variety of food sources and may thus be able to meet their nutritional requirements sooner than specialist feeders, but may face increased competition for resources with other generalists, decreasing overall fitness (Ali and Agrawal 2012, Devictor *et al.* 2008, Hanski *et al.* 1991). Generalists will thus be able to better adapt to changing environments. Specialist species may experience decreased access to resources, but will obtain a higher nutritional value from their food sources, to meet their requirements.

Specialist species may also face increased competition from other specialists, as well as from generalists (Ali and Agrawal 2012, Devictor *et al.* 2008, Hanski *et al.* 1991). Specialist species are more likely to go extinct as they are unable to adapt to change as generalist species. An example of a generalist feeder is the raccoon (Beasley *et al.* 2011, Eagan *et al.* 2011), which feeds on berries, small animals, eggs and plant matter. Examples of specialist feeders are panda bears and koala bears, who feed almost exclusively on bamboo and eucalyptus leaves, respectively (Marschner *et al.* 2017, Schaller 1985, Shipley *et al.* 2009).

Arthropods that colonize carrion can also be classified as specialists or generalists. Many arthropod species that are not directly associated with carrion-feeding are often found around a carcass, such as flesh flies (Diptera: Sarcophagidae) and house flies (*Musca domestica*). Flesh flies are generalists in that they are not limited to decomposing matter, and will utilize live animal matter or feces as a feeding and oviposition substrate (Bänziger and Pape 2004, Pape 1996). House flies are found in association with carrion as well as common waste, feces, and animal farms, classifying them as generalists as well (Hafez 1950, Levine and Levine 1991). Soldier flies are generalists as the larvae are able to feed and persist on decomposing organic substrates, feces, and carrion (Diener *et al.* 2009, 2011, Liu *et al.* 2008, Paz *et al.* 2015, Sheppard *et al.* 2002). On the opposite end of the feeding spectrum, blow flies (Diptera: Calliphoridae) feed almost exclusively on animal organic matter (both decomposing and living) and feces, making them specialist feeders (Dethier 1976, Greenberg 1991). The level of feeding specialization is highly dependent on the scale of resource being consumed.

1.5 Community diversity and relationships on carrion

Biological communities utilizing carrion are hugely abundant and diverse. As such, numerous ecological relationships arise during the process of decomposition. The presence of an array of flies, beetles, microbes, and vertebrate scavengers give rise to a complex variety of relationships, some beneficial and some detrimental, but all contributing to the decomposition and ultimate nutrient recycling of the carrion resource.

The most commonly observed relationships on carrion are (i) coexistence, whereby non-predatory arthropods and non-toxic microbes utilize the resource without affecting the life histories of other coexisting species (Ives 1991); (ii) predation, which can be intra-specific, such as third instar *Chrysomya rufifacies* (Macquart) and *Chrysomya albiceps* (Wiedemann) (both Diptera: Calliphoridae) larvae feeding on first and second instar larvae of the same species (Shiao and Yeh 2008), or inter-specific, such as many species of rove beetles (Coleoptera: Staphylinidae) that feed on fly larvae (Kočárek 2003); (iii) exclusion, whereby individuals produce compounds that inhibit other species from utilizing the resource (Janzen 1977). These compounds may be deterrents, which overwhelm the odors emitted by the carcass and make it seem unattractive to species wanting to colonize, or toxic, killing off any species consuming them, thus excluding them and permitting exclusive utilization of the resource by the microbial community (Janzen 1977); and (iv) vertebrate scavenging, which represents a unique function on a carrion resource. Vertebrate scavengers may alter the community composition entirely in a very short time period by removing large portions of the species present when they locate a resource (Kneidel 1984), but may also facilitate the utilization of the carcass by other organisms, by opening up the cavities and exposing larger portions of the resource for colonization and consumption (Kneidel 1984).

In addition to the biotic factors influencing community structure on carrion, numerous abiotic factors can also contribute to the species surviving on a resource. Sudden fluctuations in temperature can kill off a large portion of colonizing species (Niederegger *et al.* 2010); extensive rainfall can result in significant loss of both number and diversity of arthropods colonizing carrion (Archer 2004); and wind speed may prevent adult colonization of the resource (Taleb *et al.* 2016). Furthermore, extended periods of drought, and snow fall or freezing can also significantly delay arthropod colonization of carrion resources (Catts and Haskell 1990). Terrestrial arthropods also face restrictions in accessing remains located in aquatic environments, with only a selection of aquatic arthropods being able to utilize the carrion resource (Haskell *et al.* 1989).

1.6 Volatile Organic Compounds (VOCs)

Insects across multiple orders rely largely on chemical cues to locate resources in the form of mates, habitats and food sources (Nordlund and Lewis 1976). These chemical cues, which alter the behavior of the insect and direct it in a certain way, are collectively known as semiochemicals (Nordlund and Lewis 1976). Of particular interest in decomposition ecology are a class of semiochemicals called allelochemicals, further classified as apneumones: Substances emitted from a non-living organism which benefit the receiver, but not the emitter (Nordlund and Lewis 1976). These various classes of semiochemicals, particularly those that evaporate readily at room temperature, are commonly defined by chemical ecologists as volatile organic compounds (VOCs) (Cicolella 2008). While these compounds are not detectable in the short-term by human olfactory receptors, arthropods chemoreception is much more acute and can detect even trace amounts of these volatile molecules (LeBlanc and Logan 2010). The concentration and direction of origin of these molecules will guide the insect to the resource for utilization (Frederickx *et al.* 2012). Insects are sensitive to individual compounds and are able to respond to the emission of specific VOCs, whereas humans exhibit a tendency to respond to bouquets of chemicals (Kasper *et al.* 2012, LeBlanc and Logan 2010). This sensitivity contributes to an arthropods ability to detect and arrive at a carrion resource within minutes after death (Tomberlin and Adler 1998).

VOCs are isolated using gas chromatography/mass spectrometry (GC/MS) techniques and can be compared to known libraries for identification (Forbes and Perrault 2014). Common compounds associated with carrion decomposition include, among others, dimethyl disulfide (DMDS), dimethyl trisulfide, dimethyl tetrasulfide, indole, phenol, and an array of carboxylic acid derivatives (Dekeirsschieter *et al.* 2009, Haubruge *et al.* 2017, Jürgens and Shuttleworth 2016, Kasper *et al.* 2012).

Once a carrion resource has been accepted by an insect, it may be used for feeding (as a protein meal to stimulate offspring production) (Rivers *et al.* 2014), refuge/habitat (Arnaldos *et al.* 2004), or as an oviposition site in the case of gravid females (Archer and Elgar 2003). Adults utilizing the resource for food spend a significant amount of time moving over the surface of the carcass, feeding on exposed areas (Rivers *et al.* 2014). During the feeding process, salivary excretions are released onto the surface of the carcass, as well excretory products (Rivers *et al.*

2014). Once eggs hatch and larvae begin feeding, they further contribute to the excretions and secretions already present the carcass (Barnes *et al.* 2010). The addition of these compounds to the volatile compounds emitted by carrion can act as both stimulant or deterrent signals for other arthropods, and change the volatile profile (Barnes *et al.* 2010).

1.7 Olfaction in insects

Insect olfaction is critical for survival, mate finding, predator avoidance and oviposition (Gadenne *et al.* 2016). Insects may exhibit a number of behaviors associated with odors in their environment, including directed movement toward or away from a particular odor (Visser 1986), or signaling conspecific individuals to alert them to the scent (Pankiw 2004). While insects rely heavily on olfaction for survival, not all insects of the same species will respond to a particular odor in the same way. Odor reception may be dependent on physiological status. For example, sexually mature insects may respond to an odor not detected by immature individuals, while an insect that has not taken a meal will respond to a food odor, whereas a fed insect may ignore this odor (Gadenne *et al.* 2016). As such, insects may exhibit olfactory plasticity, whereby they modify their olfactory responses based on environmental cues and physiological status by modulation within the sensory system (Gadenne *et al.* 2016).

To detect even the smallest concentrations of odors in the air, insects have developed a sophisticated sensory system of olfactory receptor neurons (ORNs) housed in the antennae (De Bruyne and Baker 2008, Rützler and Zwiebel 2005). In conjunction with cuticular pores and odorant binding proteins (OBPs), odorant molecules are detected in the environment and converted into an action potential, triggering a response to the odor stimulus (De Bruyne and Baker 2008, Rützler and Zwiebel 2005).

While little is known about chemosensation in blow flies, olfaction has been extensively studied in *Drosophila melanogaster* (Krishnan et al. 1999, Stocker 1994)*, Aedes aegyptii* (Bohbot *et al.* 2007, Zwiebel and Takken 2004), and *Glossina morsitans* (Otter and Van Naters 1992, Turner 1971, Vale *et al.* 1988). *D. melanogaster* is frequently used as a model organism to study numerous aspects of insect physiology, ecology and behavior (Beckingham *et al.* 2007, Roberts 2006), while *A. aegyptii* and *G. morsitans* are highly reliant on olfaction to locate suitable hosts and obtain blood meals necessary for survival (Cork 1996, Geier *et al.* 1996, Takken 1991, Willemse and Takken 1994). These species have also been well studied due to their economic importance as vectors of disease (Gingrich *et al.* 1982, Harrington *et al.* 2005, Hu and Aksoy 2005, Morrison *et al.* 2008).

1.8 Rats as an analogue for human decomposition

Historically, pig carcasses have been used as model organisms as a proxy for human cadavers when studying decomposition and arthropod activity (Biavati *et al.* 2010, Matuszewski *et al.* 2019, Rosa *et al.* 2011). Similarities in body composition, diet and gut microbiota (Miller and Ullrey 1987, Pond and Houpt 1978) have allowed the use of pig carcasses as their decomposition mirrors that of human decomposition processes (Payne 1965, Tomberlin and Adler 1998). However, multiple studies have used smaller organisms, such as rats (de Carvalho Moretti *et al.* 2008, Moura *et al.* 1997), rabbits (Corrêa *et al.* 2014) and dogs (Jirón and Cartín 1981) to study the processes of decomposition and associated insect activity.

Body composition between the three species (pigs, rats and humans) is fairly similar, in that there exists comparable body water, fat and protein compositions for each (Miller and Ullrey 1987, Sheng and Huggins 1979). All three species exhibit an omnivorous diet, feeding on a variety of both plant- and animal-based products (Graham and Åman 1987, Guiry and Gaulton 2016). Because of the similarity in dietary preferences, the gut microbiota of all three organisms are fairly similar (Daş *et al.* 2016, Gilbert *et al.* 2018, Graham and Åman 1987, Guiry and Gaulton 2016, Miller and Ullrey 1987), with *Escherichia coli* being the dominant gut bacterium present (Gilbert *et al.* 2018, Miller and Ullrey 1987). A comparison of pig, rat and human gut microbiota revealed numerous microbial species common to all three organisms (DeBruyn and Hauther 2017, Hyde *et al.* 2013, Kim and Isaacson 2015, Leser *et al.* 2002, Li *et al.* 2017, Morotomi *et al.* 1976). Volatile organic compound analyses between rodent and pig carcasses and human cadavers also reveal a bouquet of identical compounds (LeBlanc and Logan 2010). Predominant compounds from all three groups include phenol, indole, dimethyl disulfide, dimethyl trisulfide, and xylene (LeBlanc and Logan 2010).

While carrion decomposition has been modelled to a large extent on fetal pigs, producing distinct stages of decomposition as described by Payne (1965), Tomberlin and Adler (1998) found that the stages of decomposition of terrestrial rat carcasses followed the stages of decomposition as described on pigs by Payne (1965). By these findings, it is reasonable to assume that rats will be a suitable study organism to examine arthropod activity on carrion for decomposition purposes. While there are limitations to using rats as a proxy for human cadavers, the findings presented here will allow for explanations of broadly applicable principles of decomposition ecology not limited to human studies.

1.9 Biology of the secondary screwworm, *Cochliomyia macellaria*

Cochliomyia macellaria (Fabricius) (Diptera: Calliphoridae) is a native North American species commonly known as the secondary screwworm (Baumgartner and Greenberg 1985). The term “secondary” comes from the tendency and ability of this species to cause secondary myiasis (Baumgartner 1993). Although it is commonly regarded as an early colonizer of carrion that may arrive within minutes after carrion exposure to the environment (Byrd and Butler 1996b, Hall 2010, Tomberlin and Adler 1998, Whitworth 2006), there have been reports indicating that *C. macellaria* will only colonize a carrion resource up to 72-hours after exposure (Hall and Doisy 1993). While such reports are rare, the possibility does exist that *C. macellaria* can thus act as either a primary or secondary colonizer of carrion. This species has been known to colonize larger vertebrate remains, such as sheep, goats and horses (Denno and Cothran 1975, Deonier 1940)

Cochliomyia macellaria is a warm weather species native to temperate and tropic regions of the world (Byrd and Butler 1996b, Whitworth 2006), but can exhibit mating and oviposition behavior at temperatures as low as 14.5°C (Deonier 1940). Adult size ranges between 5-8mm, with a characteristic metallic greenish-blue color, and three longitudinal stripes on the dorsal side of the thorax (Boatright 2009). On a suitable substrate, females may oviposit between 50 to 200 eggs per clutch, measuring around 1mm each in length (Boatright 2009, Flores 2013, Greenberg 1991). Group oviposition is known to occur between females, resulting in occurrences of *C. macellaria* eggs covering large portions of the remains (Boatright 2009). Densities of more than 1000 larvae/cm^2 have been found colonizing remains, which may result in rapid consumption of the decomposing resource (Laake *et al.* 1936, Oliveira and Vasconcelos 2010, Slone and Gruner 2007). Larvae that hatch will develop through three larval stadia, reaching lengths of up to 17mm, after which they pupate and emerge as adults. The time taken by larvae to develop and reach adulthood has been found to be highly dependent on temperature (Boatright and Tomberlin 2010), as well as substrate on which the larvae feed (Boatright 2009, Boatright and Tomberlin 2010, Flores 2013). After the third larval instar, pre-pupae move up to 60cm away from the remains and pupate in the soil, between 4 and 5cm deep (Godoy *et al.* 1995).

Natural populations of this species have declined significantly since the introduction of invasive blow fly species such as *Chrysomya albiceps* and *Chrysomya rufifacies* (Wells and Greenberg 1992), which are competitors for carrion material, and are also predatory on *C. macellaria* larvae (Flores *et al.* 2017, Wells and Greenberg 1992). Predation on *C. macellaria* by predatory *Ch. albiceps* and *Ch. rufifacies* has been shown to occur almost exclusively during the third instar, reducing populations by up to 75% in laboratory studies (Wells and Greenberg 1992).

1.10 Biology of the black soldier fly, *Hermetia illucens*

Hermetia illucens (L.) (Diptera: Stratiomyidae), commonly known as the black soldier fly, is a large (18 - 22mm) fly that commonly breeds in decaying substrates, including fruits and vegetables, damp grains, and decomposing vertebrate matter (Bondari and Sheppard 1981, Čičková *et al.* 2015, Diener *et al.* 2011, Sheppard *et al.* 1994). This species is common in tropical and subtropical regions of the world, and thrives in moist environments (Bondari and Sheppard 1981, Tomberlin *et al.* 2002), flourishing at warmer temperatures (Tomberlin and Sheppard 2002a). Due to their tropical tendencies, much of the current research surrounding *H. illucens* has been focused on maintaining year-round colonies in fluctuating temperatures (Holmes *et al.* 2012, 2013, Sheppard *et al.* 2002, Tomberlin *et al.* 2009, Tomberlin *et al.* 2002).

For successful mating and oviposition, natural light is required, as artificial light hinders mating and oviposition to a maximum of 61% (Tomberlin and Sheppard 2002, Zhang *et al.* 2010). Other factors influencing the mating and oviposition success of the black soldier fly include humidity, time of day, and temperature. Tomberlin and Sheppard (2002) found that 80% of egg clutches were deposited when humidity was 60% or greater, and that that peak mating occurs earlier in the day (before 15h00), with mating behavior decreasing as the day progresses. All oviposition occurs at temperatures exceeding 26°C (Tomberlin and Sheppard 2002), with females ovipositing in crevices near the food source for emerging larvae (Booth and Sheppard 1984). At 24°C, larvae emerge within 102 to 105 hours (approximately four days, dependent on temperature). The average life cycle of *H. illucens* from emergence to death ranges between 40 to 43 days but can reach up to four months in unfavorable conditions (Furman *et al.* 1959, Tomberlin *et al.* 2002). Young larvae are frail and can easily succumb to death when excess larval diet is present, as this facilitates the presence of molds (Tomberlin *et al.* 2002).

Adults have no functioning mouthparts and thus do not actively feed, nor are they known to transmit any diseases or bite, although they can cause facultative human myaiasis (James 1947, Martínez-Sánchez *et al.* 2011). After eclosion, mating usually occurs within two days (Tomberlin and Sheppard 2002), with oviposition occurring around four days after eclosion (Tomberlin and Sheppard 2002, Tomberlin and Sheppard 2001). Females lay a single clutch of eggs, in numbers ranging from 206 to 639 eggs per clutch (Tomberlin *et al.* 2002). Because adults do not feed, larvae accumulate sufficient nutrients and subsequently, body fat, during larval feeding stages to survive as non-feeding pre-pupae, pupae, and adults (Nguyen *et al.* 2015, Sheppard *et al.* 2002), and have also been found to survive longer when provided with water (Tomberlin *et al.* 2002).

Though mostly associated with waste reduction and management (Sheppard *et al.* 1994), *H. illucens* has gained popularity as a study organism due to being an alternative food source for other organisms (Bondari and Sheppard 1981, Newton *et al.* 1977, St-Hilaire *et al.* 2007), as well as an important indicator species in forensic entomology (Pujol-Luz *et al.* 2008, Tomberlin *et al.* 2005, Vanin *et al.* 2011). Even though they thrive in tropical climates, shorter lifespan and smaller adults have been associated with increasing temperature (Tomberlin *et al.* 2009). *H. illucens* have upper developmental limits of between 30 and 36°C, but colonies sustained beyond 36°C is beyond their optimal range (Tomberlin *et al.* 2009).

The black soldier fly has been found to be a late colonizer of carrion, with larvae and pre-pupae in Europe reported on human remains in the advanced stages of decay, between 20- and 30-days post mortem, although the species has been observed within the first week post mortem (Lord *et al.* 1994, Martínez-Sánchez *et al.* 2011, Myers *et al.* 2008, Sheppard *et al.* 1994, Tomberlin *et al.* 2005, Vanin *et al.* 2011), suggesting a highly variable time of colonization.

Larval development of *H. illucens* has been examined multiple times on a variety of substrates (Bondari and Sheppard 1981, Cammack and Tomberlin 2017, Gobbi *et al.* 2013, Holmes *et al.* 2013, Tomberlin *et al.* 2002), temperatures (Harnden and Tomberlin 2016, Tomberlin *et al.* 2009), at various degrees of relative humidity (RH) (Holmes *et al.* 2012), as well as multiple other traits relating to life history (Booth and Sheppard 1984, Cammack and Tomberlin 2017b, Martínez-Sánchez *et al.* 2011, Nguyen *et al.* 2015, Nguyen *et al.* 2013). Because it is a late colonizer of carrion, and has been found to colonize decomposing matter, including kitchen waste, swine and poultry manure, and chicken feed, most of which may be considered "aged resources" due to their nature of collecting over time, it can be inferred that this species develops preferentially in an older substrate as opposed to a fresh substrate.

1.11 Overview and objectives

This research aimed to identify a link between VOCs associated with carrion as a mechanism regulating behavioral responses of a specialist and generalist competing for these resources. For the purposes of this work, a specialist is defined as a species with a narrow feeding breadth, relying on carrion and feces, and a generalist is defined as a species with a wide feeding breadth, utilizing carrion, decaying plant matter, feces and combined food waste. Using rats as a proxy for human decomposition, this research aims to describe the volatile emitted by rat carcasses over time, and evaluate the behavioral responses of two fly species associated with carrion to carcasses of varying ages. These results will provide insight into the utilization of carrion by species exhibiting different feeding strategies, as well as evaluating drivers of arthropod succession on carrion.

The specific objectives of this study are to:

i. **Describe the VOCs emitted from both colonized (by *C. macellaria*) and uncolonized rat carcasses over time.**

   Rationale: Insect attraction to any resource is governed largely by olfactory cues and responses (LeBlanc and Logan 2010). Carrion-colonizing arthropods can arrive at a resource within minutes after environmental exposure; an activity largely driven by the VOCs produced by the resource (Byrd and Butler 1996, LeBlanc and Logan 2010). VOCs produced by decomposing animal matter are largely generated by physiological processes (i.e. tissue breakdown) and microbial action (Anderson 2016, Forbes and Perrault 2014, Perrault *et al.* 2015). When examining a carrion resource, the presence or absence of microbes and/or arthropods can be an indicator of environmental quality, and may also indicate disturbances (Liu *et al.* 2016). The different VOCs produced serve as cues to attract different groups of arthropods to the resource at different times (Barnes *et al.* 2010). This objective was to differentiate the VOCs produced during the decomposition process, as well as quantify the differences in VOC's (if any) when a primary colonizer is actively feeding on the resource. Compounds of biological significance will be examined for their contribution to the VOC profile emitted to the environment in attracting an arthropod colonizer. Hypotheses tested include:

$H1_0$: No change in VOC profile over time.

$H1_A$: Significant changes in composition and concentrations of compounds in VOC profile over time, with an increase in decomposition-associated compounds with time.

$H2_0$: No difference in VOC profile between colonized and uncolonized rat carcasses.

$H2_A$: Significant difference in VOC profile between colonized and uncolonized rat carcasses, with colonized carcasses releasing decomposition-associated compounds earlier than uncolonized carcasses.

ii. Examine the attraction of a specialist feeder, secondary screwworm, *Cochliomyia macellaria,* to fresh vs aged carrion.

Rationale: As a primary colonizer, *C. macellaria* often arrives at a carrion resource within minutes after environmental exposure (Byrd and Butler 1996). This species is a specialist feeder and is heavily reliant on decaying vertebrate matter for larval survival, perishing in the absence of a suitable resource (Hanski *et al.* 1991). As such, the immatures of this species are limited both spatially and temporally to the resource they are colonizing. Being one of the first species to arrive, *C. macellaria* are often used to estimate TOC in medicolegal entomological investigations (Owings *et al.* 2014). Historically, there have been reports indicating that this species will only colonize carrion during the later stages of decomposition, thus can be classified as a secondary colonizer as well (Hall and Doisy 1993). The purpose of this objective is to clarify the preferred oviposition sites of *C.*

macellaria, as well as to determine whether the presence of a primary colonizer affects the oviposition site choice. To investigate this objective, I will examine multiple hypotheses:

H1$_{0}$: *Cochliomyia macellaria* will not show any oviposition preference between uncolonized variably aged carcasses.

H1$_{A}$: *Cochliomyia macellaria* will exhibit an oviposition preference to a fresh carcass due to decreased interspecific competition from microbes, as well as the propensity of this species to being a primary colonizer and arriving at a carcass within minutes after death.

H2$_{0}$: *Cochliomyia macellaria* will not show a preference between previously colonized variably aged resources.

H2$_{A}$: *Cochliomyia macellaria* will exhibit an oviposition preference to a previously colonized fresh carcass, based on its propensity to being a primary colonizer and establishing on the carcass during the earlier stages of decomposition to avoid both inter- and intraspecific competition.

iii. Examine the attraction of a generalist feeder, black soldier fly, *Hermetia illucens,* to fresh vs aged carrion.

Rationale: *Hermetia illucens* has historically been found colonizing vertebrate remains at various stages of decomposition; but, there are conflicting reports as to whether this species exhibits a preference for fresh or aged carrion (Dunn 1916, Lord *et al.* 1994, Pujol-Luz *et* *al.* 2008, Tomberlin *et al.* 2005). Being described as a primary and secondary colonizer means that larvae will develop on resources of different ages and thus different nutritional composition and quality. *H. illucens* is a generalist feeder and is thus more likely to be adaptable and develop successfully on both carrion types (Bondari and Sheppard 1981). A wide diet breadth allows for utilization of multiple resources, dependent on availability (Bondari and Sheppard 1981). Such decisions, as well as the presence of other species consuming the resource, can impact offspring survivorship (Brundage *et al.* 2014). Deciphering the cues, in conjunction with objective 1, regulating colonization by *H. illucens* could provide insight as to the mechanisms regulating arthropod succession patterns on such resources. This objective is to characterize the preference of *H. illucens* colonization and oviposition between a fresh and aged resource, to establish (a) the drivers behind colonization of a resource in this species, largely based on volatile emissions and (b) to clarify the accuracy of its' utilization as an estimator of TOC. Multiple hypotheses will be investigated here:

$H1_0$: *Hermetia illucens* will show no oviposition preference to fresh or aged resources.

$H1_A$: *Hermetia illucens* will exhibit a preference in oviposition site to an aged resource as a result of an increasing VOC profile with time, in addition to increasing microbial activity with time.

$H2_0$: *Hermetia illucens* will show no preference in oviposition between fresh and aged resources, and Gainesville diet.

$H2_A$: *Hermetia illucens* will exhibit a preference of oviposition site on Gainesville diet due to the digestibility and homogeneity of the substrate.

H3$_0$: *Hermetia illucens* will show no preference in oviposition site between fresh and aged resources previously colonized with larvae of a primary colonizer (*C. macellaria*).

H3$_A$: *Hermetia illucens* will exhibit a preference in oviposition site to previously colonized aged resources, based on the presence of predigested material as a result of feeding activity of the primary colonizer larvae.

H4$_0$: *Hermetia illucens* will show no preference in oviposition site between colonized fresh and colonized aged resources, and Gainesville diet.

H4$_A$: *Hermetia illucens* will exhibit an oviposition preference to Gainesville diet due to digestibility and homogeneity of the substrate.

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CHAPTER II

VOLATILE ORGANIC COMPOUNDS IN VARIABLY AGED CARRION IMPACTED BY THE PRESENCE OF THE PRIMARY COLONIZER, *COCHLIOMYIA MACELLARIA* (DIPTERA: CALLIPHORIDAE)

The attraction and colonization of vertebrate remains by carrion arthropods is a process largely governed by olfaction. As remains decompose, they emit a bouquet of volatile organic compounds (VOCs), which originate from endogenous and exogenous microbes surrounding the carcass or the carcass itself. The ability to detect and respond to these VOCs, even at low concentrations, may be critical to the survival of carrion-dependent arthropods. The composition and concentration of VOCs changes based on the microbial species presence and viability, the presence or absence of arthropods, as well as on temperature. Gas chromatography-mass spectroscopy analysis was used to identify and classify volatile emissions from insect colonized and uncolonized rat carcasses, as well as a standard Gainesville diet, over a ten-day period. There were significant differences in composition and abundance of compounds present in each treatment, with significant effects of day and treatment on compound composition. Notable indicator compounds included indole, dimethyl disulfide, phenol, and dimethyl trisulfide. A high compound richness, and a low compound diversity, were detected over the ten-day period. Approximately 68.7% of the variation between treatments was explained by multivariate analysis, namely permutational analysis of variance (PERMANOVA) and non-metric multidimensional scaling (NMDS). The indicator compounds detected across all treatments were found to be of microbial origin, highlighting the importance of microbes in decomposition processes and arthropod attraction to carrion. The results of this study provide insight into the changes in decomposition VOCs over time, with an explanation of compounds in high concentration known to be attractive to carrion-colonizing arthropods.

2.1 Introduction

The process of decomposition is one that has been well studied and documented from an ecological standpoint (Barton *et al.* 2013, Benbow *et al.* 2015, Horenstein *et al.* 2010). Historically, much focus has been on the physiological factors governing this process, including the stages of decomposition, the influence of arthropods, as well as the effects of abiotic influences, such as temperature, precipitation, and burial or concealment (Archer 2004, Forbes and Carter 2016, Payne 1965, Pechal *et al.* 2014). As decomposition of carrion progresses, a complex array of chemical reactions takes place, resulting in microbial proliferation, odor emission and physiological changes to the carrion itself (Carter *et al.* 2015, Forbes and Perrault 2014, Paczkowski and Schütz 2011, Pechal *et al.* 2013). Although the resource is ephemeral, a myriad of changes take place on the resource in a very short space of time. Potentially, within the first few hours, blow flies (Diptera: Calliphoridae) are attracted to the carrion resource, and begin colonization (Carvalho *et al.* 2000). As decomposition progresses, various other organisms begin to colonize the remains, including numerous beetles (Coleoptera), moths (Lepidoptera), wasps and bees (Hymenoptera), and other fly families (Anderson and VanLaerhoven 1996).

The initial attraction to vertebrate remains is thought to be governed largely by olfactory cues (LeBlanc and Logan 2010). These olfactory cues exist as volatile organic compounds (VOCs) emitted from the carrion resource. VOCs are primarily of microbial origin, produced by various aerobic and anaerobic microbes present on the carcass, in the soil, and brought in from external sources (such as vertebrate and invertebrate scavengers) (Cammack *et al.* 2015). Cadaveric VOCs have been documented from human (Statheropoulos *et al.* 2005, Vass *et al.* 2008), swine (Dekeirsschieter *et al.* 2009, Perrault *et al.* 2015), bovine remains (Dubie *et al.* 2017), as well as those of smaller mammals, such as mice (Paczkowski *et al.* 2012), lamb, and roe (Rosier *et al.* 2017). Identification, isolation, and classification of these VOCs, as well as the microbes responsible for their production, has widespread applications, including determination of the post-mortem interval (PMI) (Metcalf *et al.* 2013) and cadaver dog training (Rust *et al.* 2016). As microbes proliferate on the remains, and microbes, arthropods, and scavengers compete for the carrion resource, the composition and concentration of VOCs change based on who is present during that specific time (Carter *et al.* 2015, Weatherbee *et al.* 2017). Changes in VOCs may influence arthropods and scavengers in different manners, with some compounds serving as attractants (Brundage *et al.* 2017, Liu *et al.* 2016), and other serving as repellents (Liu *et al.* 2016). The attraction or repulsion ability of VOCs to an arthropod also depends on the physiological status and sex of the arthropod. Liu *et al*. (2016) found that in *Lucilia sericata* (Meigen) (Diptera: Calliphoridae), indole was an attractant at higher concentrations, but served as a repellent in lower concentrations, while dimethyl disulfide (DMDS) elicits an attractive response to gravid females in higher doses and males are attracted to this compound at lower doses (Liu *et al.* 2016).

While the scope of compounds will vary between organisms, based partially on diet and exposure to different environmental elements, numerous common compounds have been identified as being associated with vertebrate remains. These include DMDS, dimethyl trisulfide (DMTS), dimethyl tetrasulfide, indole and phenol, each of which is been shown in its highest concentration at different stages during decomposition (Forbes and Perrault 2014).

A grey area of VOC analyses, and one which largely governs arthropod attraction, is understanding the changes in VOC profiles over time under standard conditions, and how the composition changes in the presence or absence of primary colonizers. Few studies have definitively classified how the VOCs emitted from vertebrate remains change over time (Forbes *et al.* 2014, Stadler *et al.* 2015). Understanding the chemistry of death through VOC emissions after environmental exposure, prior to and during arthropod colonization, is of key interest to forensic entomologists as research begins to resolve the pre-colonization interval and determine what is driving attraction of arthropods to decaying vertebrate remains. The present study evaluated the changes in volatile profiles of carrion over time, both in the presence and absence of a primary colonizer, *Cochliomyia macellaria* (Fabricius) (Diptera: Calliphoridae), and compared these profiles to plant-based decaying matter profiles which are preferred by a secondary colonizer, *Hermetia illucens* (L.) (Diptera: Stratiomyidae).

2.2 Methods

2.2.1 *Cochliomyia macellaria* colony maintenance and egg acquisition

Cochliomyia macellaria was maintained at the F.L.I.E.S Facility, Texas A&M University, College Station, TX, and has been maintained for >30 generations, with wild-type individuals introduced periodically. Adults were maintained in standard insect rearing cages (BugDorm$^{©}$; 30 x 30 x 30 cm) at 27°C, a 14:10 L:D cycle and 60% RH. Cages were supplied with a mason jar containing water, along with a 1:1 honey and water solution and a 1:1 powdered milk and sugar mixture. To stimulate oviposition, adult flies were provided with a bovine blood meal (± 10 ml) five and seven days after emergence. Nine days after emergence, adults were supplied with ± 50 g bovine liver placed on a paper towel in a 90 ml plastic cup for a period of 8 h. Eggs were separated from liver and placed in a mason jar with vermiculite for rearing. After hatching, larvae were provided with bovine liver *ad libitum* until pupation. Upon emergence, adults were transferred to BugDorm$^{©}$

cages. *Cochliomyia macellaria* voucher specimens were submitted to the TAMU Insect collection under voucher number 742.

2.2.2 Sample preparation & maintenance

Twenty-four hours prior to the first collection of VOCs, six aluminum foil bread pans (20 x 9.5 x 6 cm) were wiped clean with a 10% Alconox™ solution, followed by a 70% ethanol, acetone, and then allowed to air dry inside a standard fume hood (~24°C, natural light ~12:12 L:D; 65% RH), which was wiped clean using the same protocol. For uncolonized treatments, four frozen rats (*Rattus rattus* Linnaeus) (RodentPro®; 175-275 g; all male) were removed from a freezer and thawed in Ziploc bags for 24 h prior to sampling.

When VOC sampling commenced, rats were weighed and placed into the foil containers. Empty foil containers were set up as controls. VOCs were sampled at the commencement of this study as described in the next section. After VOCs collection, the foil covering was removed from the containers, and these containers were placed into larger, plastic containers (30 x 15 x 12 cm) inside the fume hood. These plastic containers had windows (10 x 5 cm) on the sides which were sealed with WipeAll coverings, and covered and sealed with a layer of aluminum foil. The above procedure was observed after each sample was collected for a period of ten days.

For colonized treatments, rats were thawed as described above. On the day of commencement, four thawed rats were inoculated with 0.3 g freshly oviposited (<8 h) *C. macellaria* eggs each, split between the ears, mouth, and anal region. In addition, uncolonized rats were included, as well as empty foil containers, as controls. For this treatment, *C. macellaria* larvae were allowed to develop undisturbed for the ten-day period. During post-feeding, larvae were able to escape the foil container and pupate in playpen sand (Quikrete Premium play sand; Quikrete, Atlanta, GA) placed into the plastic containers. An additional treatment of standard Gainesville diet was prepared (50% wheat bran, 30% alfalfa meal, 20% corn meal; used for house fly rearing in laboratory conditions) (Hogsette 1992, Sheppard *et al.* 2002), and sampled in the same manner, with empty containers as controls.

2.2.3 VOC sample collection & elution

Volatile samples were collected from treatments and controls every 24 h. Thirty minutes prior to sampling, the foil containers with, or without, rats were removed from the plastic containers and fume hood and left to rest in standard room conditions (24°C), thus allowing any volatile residues from the plastic containers to dissipate. After this 30 min dissipation period, foil pans were tightly sealed with a layer of heavy-duty aluminum foil, and the entry and exit sites for volatile sampling sealed with masking tape. Containers were left for one hour, to allow volatile accumulation. Once volatiles had accumulated, filter traps packed with a 30 mg HayeSep Q adsorbent (Volatile Assay Systems, Rennselaer, NJ) and charcoal filters were inserted into the entry and exit sites, and connected to an extraction pump. Volatiles were extracted for one hour at a flow rate of 1L/min. After collection, each filter was eluted for later analysis using 150 µL dichloromethane (DCM) and 5 µL n-Octane (80 ng/µL) as the internal standard (IS). Samples were kept at -20°C until processing.

2.2.4 Chromatographic analysis

Eluted volatile samples were processed at the Geochemical and Environmental Research Group (GERG) facility at Texas A&M University, College Station, TX. The gas chromatography (GC) and mass spectroscopy (MS) analytical parameters are provided in Table 2.1. The parameters utilized for this protocol have been previously used for analysis of cadaveric VOCs. Identifications of compounds were made by comparing retention times with known standards.

2.2.5 Statistical analyses

To evaluate the differences in volatile profiles between treatments, all multivariate analyses were conducted in R® (v3.5.2 – "Eggshell Igloo"; 2018) using packages vegan, MASS, devTools, pairwiseAdonis and indicspecies. Bray-Curtis distance with non-metric multidimensional scaling (NMDS) with 999 permutations was used to visualize differences in volatile profiles across treatments. NMDS methods ranks orders of data points to produce an ordination plot along two axes, and do not require linearity among variables. Permutational multivariate analysis of variance (PERMANOVA) was implemented to determine differences between treatments and across days at a significance level of $p < 0.05$. Indicator species analysis was performed to establish indicator compounds for each treatment. Mean species richness (abundance) and Shannon-Weiner diversity indices (H) were also computed over time, with a repeated-measures analysis of variance (RM-ANOVA) performed to distinguish significant differences in treatments over time.

Table 2.1: Analytical parameters for gas chromatography-mass spectroscopy (GC-MS) analysis. Numbers following GC and MS indicate model numbers of equipment used.

Autosystem GC-MS
GC 6890
Carrier gas: Helium at 1.3 mL/min
Column: Agilent DB5
30 m x 0.25 mm x 0.5 μm
Oven temperature program:
Initial temperature: 35°C during 8 min
First ramp: 4°C/min, 60°C
Second ramp: 6°C/min, 160°C
Third ramp: 20°C/min, 300°C
MS 5973
Solvent delay: 3 min
Mass scan: m/z 50-550

2.3 Results

2.3.1 Decomposition process over time

Over the ten-day decomposition period, both colonized and uncolonized rat carcasses exhibited typically described stages of decomposition, showing bloat and active decay. The uncolonized carcasses exhibited bloat from days 1-3, with fluids purging the body on day 4. Microbial growth was present on day 5, and proliferated through the end of the study period on day 10. The colonized carcasses exhibited bloat on day 2, but by day 3, larvae were actively feeding on the head and anal regions, and active decay was observed. Larvae continued to feed until approximately day 6, when wandering behavior began and larvae vacated the carcass into surrounding sand. By day 8, all larvae had vacated the carcass, with a partially skeletonized carcass remaining; however, there were still large areas of soft tissue present. The Gainesville diet began to desiccate on day 3, and by day 5 there were visible microbial bodies present over the surface of the prepared matter. The microbial bodies proliferated through the remainder of the ten-day sampling period.

2.3.2 Compound classes

A total of 76 compounds belonging to various classes were identified for analysis in this study (Fig. 2.1). The following classes were represented (in parentheses, the number of individual compounds identified within each class): acids (6), esters (2), ketones (7), aldehydes (7), alcohols (10), nitrogen-containing compounds (3), sulfur-containing compounds (6), furans (1), cyclic hydrocarbons (22) and non-cyclic hydrocarbons (11) (Fig. 2.1). Of the 76 compounds selected for analysis, 65 were identified in uncolonized carcasses, 74 in colonized carcasses, and 73 in Gainesville samples.

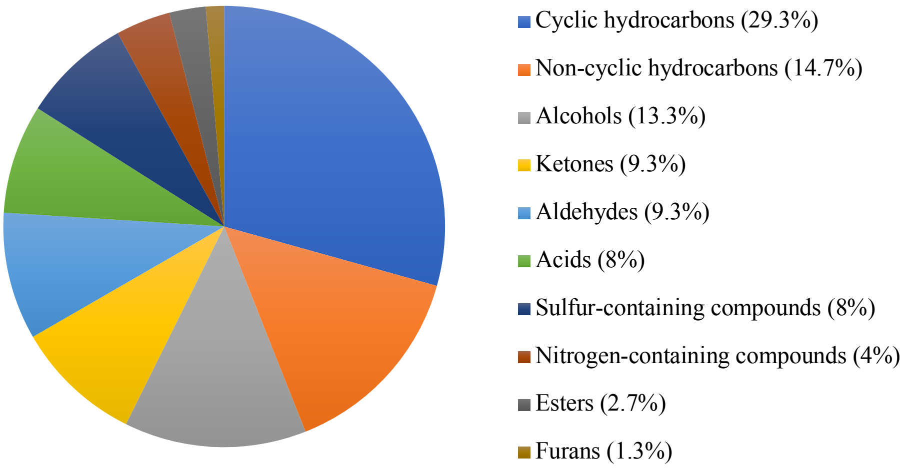

Figure 2.1: Distribution of compound classes represented across uncolonized rat carcasses, rat carcasses colonized with *C. macellaria* larvae, and standard Gainesville diet, left to decompose for ten days, under natural light cycle (~24°C, natural light ~12:12 L:D; 65% RH)

2.3.3 VOCs profile

A two-dimensional NMDS ordination analysis sufficiently described carcass volatiles between treatments, explaining 68.7% of the variation with a good fit of the model to the data (stress = 0.1533) (Dugard *et al.* 2010), and showed significant differences among the volatile profiles (Fig. 2.2). Using PERMANOVA, significant differences between the volatile profiles of all treatments were found (Fig. 2.2; Table 2.2). There was also a significant interaction of treatment and day ($p < 0.001$), and significant differences in volatile profiles across the ten-day period (Suppl. Table A2.1).

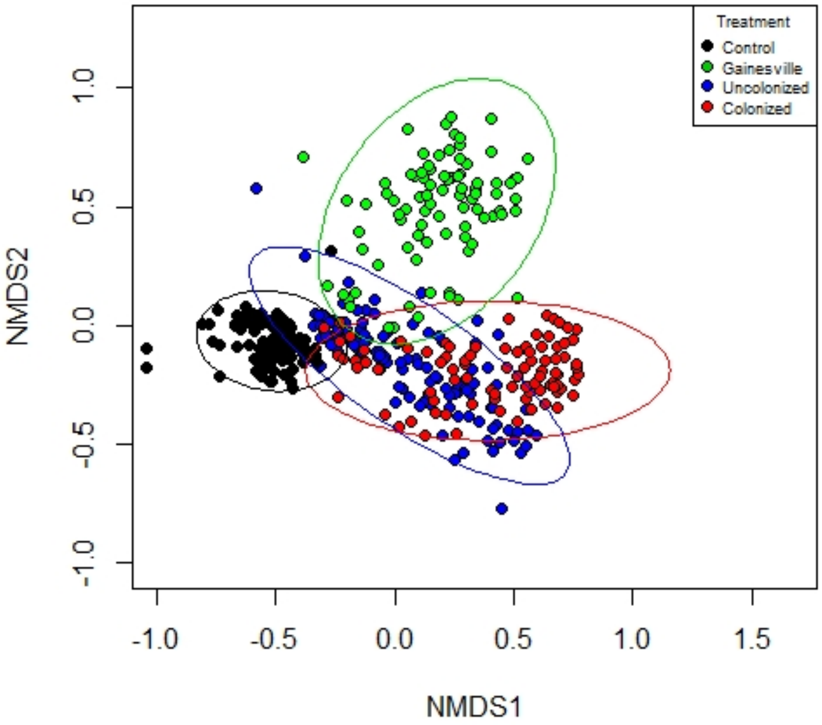

Figure 2.2: Nonmetric multidimensional scaling ordination of volatile profiles compiled from three decomposition treatments over a ten-day period. Ellipses represent the 95% confidence intervals. The decomposition treatments tested were uncolonized rats, rats colonized with *C. macellaria* larvae, and a standard Gainesville diet.

Table 2.2: PERMANOVA results testing differences in volatile composition across three treatments of decomposing matter: uncolonized rat carcasses, rat carcasses colonized with *C. macellaria* larvae, and a standard Gainesville diet. Significant differences ($p < 0.05$, Bonferroni corrections applied for multiple comparisons) are indicated by an asterisk.

Factors	**DF**	**SS**	***F***	**R^2**	***p*-value**	**Adjusted *p*-value**
Control vs Uncolonized	1	16.571	92.001	0.297	0.001	0.006*
Control vs Gainesville	1	16.955	125.332	0.390	0.001	0.006*
Control vs Colonized	1	18.830	97.453	0.332	0.001	0.006*
Uncolonized vs Gainesville	1	8.295	32.526	0.142	0.001	0.006*
Uncolonized vs Colonized	1	3.383	10.809	0.052	0.001	0.006*
Gainesville vs Colonized	1	8.978	32.151	0.156	0.001	0.006*

2.3.4 Indicator compounds

Of the 75 compounds used in analysis, two (2.7%) were found to be significantly associated with the Gainesville diet, and 18 compounds (24%) significantly associated with the colonized rat carcass (Table 2.3). There were no compounds significantly associated with the uncolonized rat carcasses. There were 12 compounds (16%) significantly associated with all three decomposition treatments (Table 2.4).

2.3.5 Compound richness

There was a general increase in the abundance of compounds over time in both the colonized and uncolonized rat carcass treatments, with a general decrease in number of compounds over time in the Gainesville diet treatment (Fig. 2.3). The number of compounds in the control samples remained fairly constant over the ten-day sampling period (Fig. 2.3). There was a significant effect of both day ($F_{10,395} = 8.9869$; $p < 0.05$) and treatment ($F_{3,395} = 408.677$; $p < 0.05$) on the compound richness observed over time, as well as a significant interactive effect of day and treatment ($F_{30,395} = 29.661$; $p < 0.05$) on the compound richness over the ten-day period.

2.3.6 Compound diversity

The diversity of compounds present in the control, Gainesville diet, and colonized rat carcass treatments remained fairly constant over the ten-day sampling period, with the exception of the Gainesville diet treatment which showed a decrease in diversity on day 1 (Fig. 2.4). The diversity of compounds in the uncolonized rat carcass treatment decreased steadily over the ten-day sampling period (Fig. 2.4). There was a significant effect of both day ($F_{1,395} = 31.5757$; $p < 0.05$) and treatment ($F_{3,395} = 4.5326$; $p < 0.05$) on the compound richness observed over time, as well as a significant interactive effect of day and treatment ($F_{3,395} = 21.5650$; $p < 0.05$) on the compound richness over the ten-day period.

Table 2.3: Indicator VOCs associated with Gainesville diet and rat carcasses colonized with *C. macellaria* larvae, left to decompose for ten days at 24°C, 65% RH and a 12:12 L:D cycle. No VOCs were significantly associated with the uncolonized rat carcass treatment.

Treatment	**Compounds**	**Indicator value**	***p*-value**
Gainesville diet	Methyl isovalerate	0.786	0.005
	1-Bromo-2-methoxybenzene	0.261	0.005
Colonized rat carcass	4-Ethyl phenol	0.965	0.005
	Indole	0.958	0.005
	Trimethyl pyrazine	0.936	0.005
	2-Octanamine	0.891	0.005
	Phenylethyl alcohol	0.879	0.005
	Naphthalene	0.868	0.005
	2-Undecanone	0.840	0.005
	Methyl pyrazine	0.815	0.005
	Hexadecane	0.812	0.005
	Hexanoic acid	0.797	0.005
	Nonadecane	0.785	0.005
	Tetradecane	0.783	0.005
	Dimethyl tetrasulfide	0.530	0.005
	4-Methyl quinazoline	0.699	0.005
	Butylated hydroxytoluene	0.657	0.005
	Diethyl phthalate	0.628	0.005
	3-Ethyl-2,5-dimethylpyrazine	0.622	0.005
	Aromadendrene	0.487	0.005

Table 2.4: Indicator VOCs associated with three decomposition treatments (uncolonized rat carcasses, rat carcasses colonized with *C. macellaria* larvae, and standard Gainesville diet) left to decompose for ten days at 24°C, 65% RH and a 12:12 L:D.

Compounds	**Indicator value**	***p*-value**
1-Octen-3-ol	0.990	0.005
2-Heptanone	0.988	0.005
2-Pentylfuran	0.986	0.005
Dimethyl disulfide	0.955	0.005
Decane	0.937	0.005
Acetophenone	0.928	0.005
Acetoin	0.905	0.005
Nonanal	0.894	0.005
1-Methyl-1H-pyrrole	0.838	0.005
1-Hexanol	0.828	0.005
2-methyl-ethyl-ester-butanoic acid	0.679	0.005
2,4,7,9-tetramethyl-5-decyn-4,7-diol	0.594	0.005

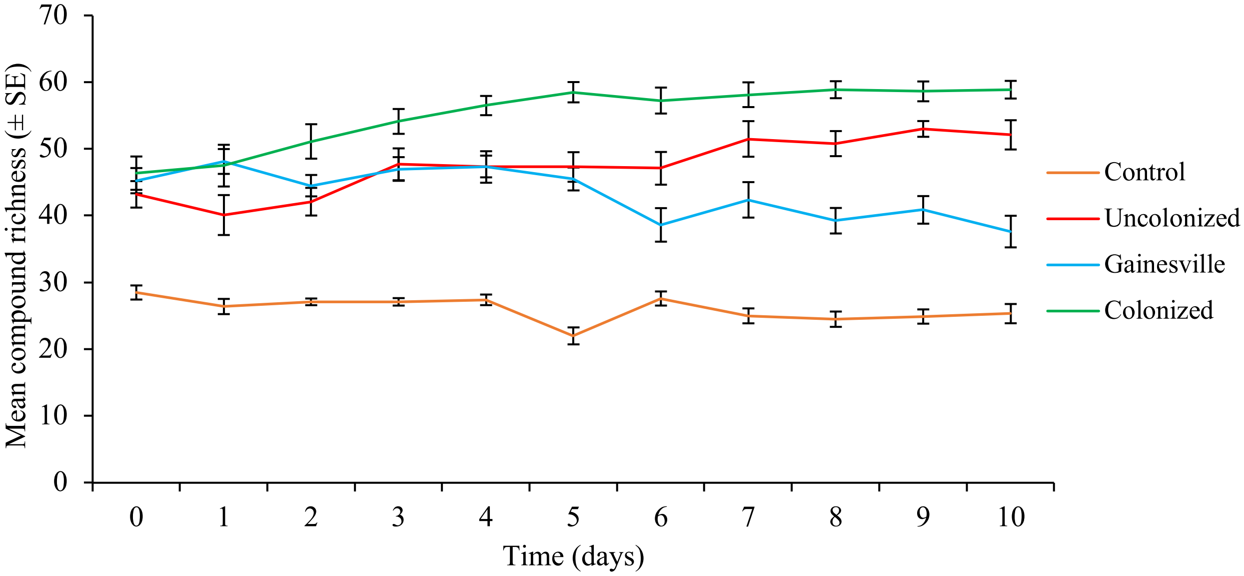

Figure 2.3: Mean compound richness (± SE) of volatile organic compounds (VOCs) analyzed from three decomposition treatments (uncolonized rat carcasses, standard Gainesville diet, rat carcasses colonized with *C. macellaria* larvae) over a ten-day period.

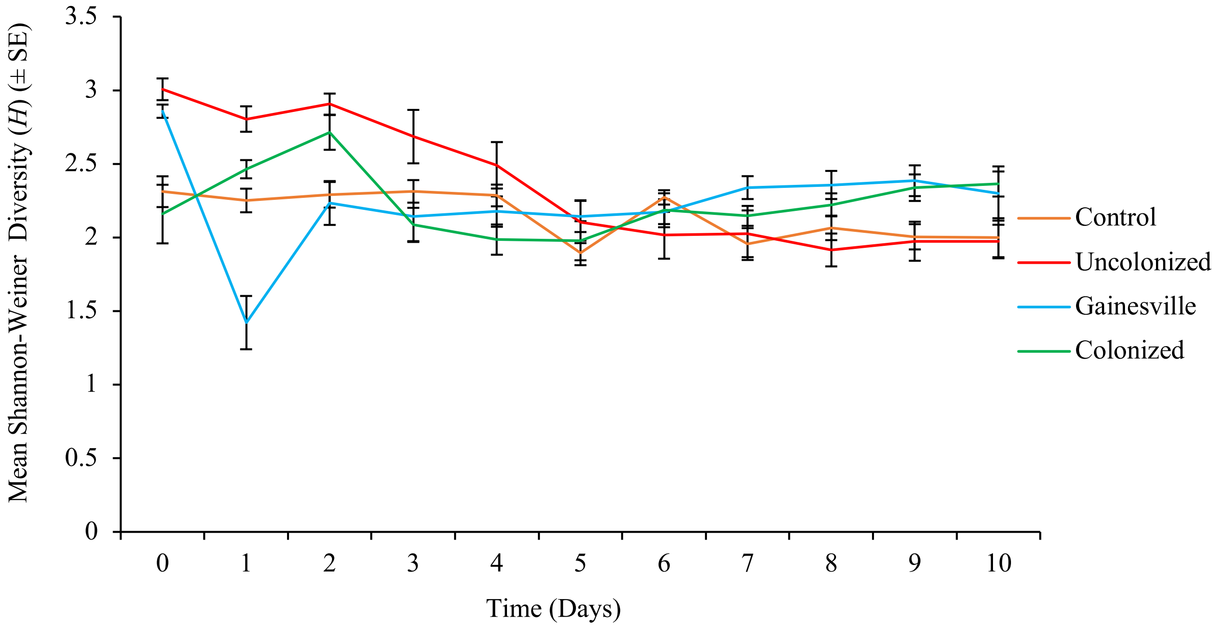

Figure 2.4: Mean compound diversity (± SE) of volatile organic compounds (VOCs) analyzed from three decomposition treatments (uncolonized rat carcasses, standard Gainesville diet, rat carcasses colonized with *C. macellaria* larvae) over a ten-day period.

2.4 Discussion

Based on our survey of the current literature, the data presented here are the first to compare decomposition VOC profiles between colonized and uncolonized carrion over time, while explaining the richness and diversity of compounds emitted during vertebrate decomposition over time.

A large portion (~65%) of the VOCs identified in this study that are associated with decomposition have been identified from pigs (Cablk *et al.* 2012, Dekeirsschieter *et al.* 2009, Irish *et al.* 2019), humans (Cablk *et al.* 2012, Statheropoulos *et al.* 2005), cows (Cablk *et al.* 2012), and chickens (Cablk *et al.* 2012). Extensive research has been conducted to evaluate the VOC emissions from porcine carrion over time, under a number of natural conditions (Cablk *et al.* 2012, Dekeirsschieter *et al.* 2009). While 75 compounds were identified and utilized for analysis in this study, and the number of VOCs identified in other studies may not match this number and there exists the possibility of more compounds being present within the samples that were in too low a concentration to detect using the methods described.

Lucilia sericata Meigen (Diptera: Calliphoridae) has been shown to be highly responsive to dimethyl disulfide and butan-1-ol (Frederickx *et al.* 2012). Although butan-1-ol was not detected as an indicator compound in this study, dimethyl disulfide was detected as a significant indicator in all three decomposition treatments. Preliminary analyses showed that DMDS begins to dominate the volatile profile at approximately day 5. As an indicator compound eliciting a strong response in *L. sericata*, a species deemed a primary colonizer, the presence of DMDS later in the decomposition process may be a contributing factor to the variability noted in arthropod succession patterns on carrion, whereby primary colonizers are often observed visiting remains during all stages of decomposition (Anderson and VanLaerhoven 1996, Arnaldos *et al.* 2004, Hall and Doisy 1993). Several unique compounds were detected in the colonized carcass, showing that once colonized by a primary colonizer, the volatile profile of the carcass changes significantly, which may affect further primary and/or secondary colonizers timing and arrival at remains. Numerous factors may be contributing to these unique compounds, including microbes introduced into the remains by feeding larvae and visiting adult flies (Ma *et al.* 2012), secretions and excretions onto the carcass (Kerridge *et al.* 2005), and the chemical reactions induced by exposure of an anaerobic biome to an aerobic environment through mechanical feeding (Crippen *et al.* 2015).

While a large portion of the compounds detected in this study were found to be of microbial origin (Suppl. Table A2.2), the physiological degradation of vertebrate tissue has also been shown to produce a number of VOCs (Forbes and Perrault 2014, Paczkowski and Schütz 2011). During the decomposition process, the degree of competition between carrion-feeding insects and microbes intensifies (Janzen 1977), in addition to the resource decreasing in physical mass as organisms continue to feed on it (Mądra *et al.* 2015). The decrease in mass also leads to a rapid depletion of nutrient-rich areas, such as internal organs (Warren and Anderson 2009). As these nutrient-rich areas become depleted, the niche availability decreases as well, and consequently, the composition of organisms, both microbes and arthropods, decreases over time (Pechal *et al.* 2013, 2014). The decrease in microbial activity on vertebrate remains results in decreased volatile production over time (Perrault *et al.* 2015). The change in community structure of the organisms utilizing carrion, as measured by distinctions in volatile production, allows for an assessment of microbial and arthropod activity, and by relating these measures to species richness and diversity analyses, a comprehensive evaluation of the interactions between microbes, arthropods, and their associated volatile production on carrion, can be conducted.

The Gainesville diet treatment was included here as a comparative treatment, to compare a decomposing plant treatment to vertebrate remains. The Gainesville diet was developed for rearing house flies *Musca domestica* Linnaeus (Diptera: Muscidae), and consists of alfalfa meal, corn meal and wheat bran (Hogsette 1992). This diet can be used for the laboratory rearing of black soldier fly *Hermetia illucens* Linnaeus (Diptera: Stratiomyidae) (Sheppard *et al.* 2002), a species that has also been recorded as being associated with carrion (Dunn 1916, Lord *et al.* 1994, Martínez-Sánchez *et al.* 2011). The detection of numerous compounds commonly associated with carrion in the Gainesville treatments may explain the attraction of this species to carrion, during both the early and later stages of decomposition (Lord *et al.* 1994, Tomberlin *et al.* 2005). Leaf litter, and the resulting plant biomass that accumulates through decomposition of plant matter, can vary substantially in chemical composition. The ratios of carbon, nitrogen and phosphorus (C:N:P) from decomposing plant matter can be highly variable, as can the composition and community structure of both microbial and invertebrate decomposers, which have specific nutritional requirements (Gessner *et al.* 2010, Martinson *et al.* 2008). Detritivores have been shown to be limited by the P and N content of their food sources (Martinson *et al.* 2008), which may affect performance on a variety of measures (Berner *et al.* 2005, Zehdner and Hunter 2009). In addition, compounds such as cellulose and lignin present in many plant tissues are not readily digestible or broken down by many invertebrates (Fioretto *et al.* 2005), which may substantially influence the composition of arthropods consuming the decaying matter (Waksman 1940). By contrast, the chemical composition of animal tissue is more stable as the time of death, but trace elements change in concentration over time, which may contribute to successional patterns observed on carrion (Parmenter and MacMahon 2009). The differences in chemical composition of plant and animal matter result in variable utilization of each resource by invertebrates and microbes, and by including the Gainesville diet in this study as a comparative control, the role of microbes in volatile production throughout the decomposition process is highlighted.

The indicator compounds detected in these analyses are of microbial origin, and have been found to have various functions, including nematocidal activity, microbial inhibition, and insect attractants (Suppl. Table A2.2). The influence of microbes in the attraction of arthropods has, in recent years, become the subject of extensive investigation (Crippen *et al.* 2015, Jordan and Tomberlin 2017, Pechal *et al.* 2013). The classification of compounds of microbial origin as indicator compounds in the present study highlights the role of carrion-associated microbes in arthropod attraction to remains, as well as the role these microbes serve in maintaining the digestibility of the remains, by other microbes, arthropods, and vertebrate scavengers by inhibiting certain microbes and producing compounds to specifically attract arthropods.

While the data presented here allow for a classification of the diversity and abundance of VOCs associated with carrion over time, the methods utilized in this study presented the potential for cross-sample contamination, explaining the high abundance of compounds present in the control treatments, as well as the variation observed in these samples. During the resting period described, all samples were left unsealed in the same room before being sealed. VOCs emitted by decomposition treatments may have become trapped in the control samples upon sealing with aluminum foil, and subsequently erroneously detected in the GC/MS analysis. Additionally, the diet of the rats prior to death is unknown, as well as whether there were stomach contents present, both of which may have influenced the microbial community composition present, and thus, the VOCs detected. Freezing of a substrate has also been shown to significantly alter the microbes present within the substrate (Flores *et al.* 2015), thus the VOC profiles obtained from this study may not be a true reflection of the microbially-derived VOCs in a recently killed organism.

Future work should aim to swab rat carcasses with every sample, and culture the swab on agar. This process will allow analysis of the change in microbial community over time, and in conjunction with the change in volatile profile, establish an association with which microbes are dominant with which indicator compounds, highlighting the biological role of microbes in the VOC bouquet produced. Furthermore, indicator compounds for each treatment, both in isolation and in various combinations, can be used to establish their exact role in arthropod attraction to carrion remains. Such results may elucidate the role of colonization of remains on the arthropod attraction to carrion, both for conspecific and heterospecific colonization and attraction. A similar study using different primary colonizer species may also be conducted, establishing the variation in VOC bouquets produced by various species.

The results of the present research highlight the variation in VOCs associated with decomposition of vertebrate remains over time, and emphasize the importance of microbial communities in the production of these VOCs. These data begin to decipher the mechanisms regulating insect behavior in response to carrion resources, by characterizing the odor plumes associated with the presence and absence of primary carrion colonizers. The classification and quantitation of VOCs associated with carrion may lead to deciphering the drivers of variation observed in arthropod succession patterns on carrion.

2.5 References

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CHAPTER III

INFLUENCE OF SUBSTRATE AGE ON OVIPOSITION BEHAVIOR OF A SPECIALIST CARRION COLONIZER, SECONDARY SCREWWORM, *COCHLIOMYIA MACELLARIA* (FABRICIUS) (DIPTERA: CALLIPHORIDAE)*

The location and consumption of carrion by arthropods is a process that can be potentially distinguished temporally based on the makeup of the associated community. In fact, succession on carrion is a continuum of different generalist and specialist arthropods entering and leaving the system. Blow flies commonly associated with vertebrate remains are considered specialists due to their reliance on these materials as a source of food for offspring and protein for gravid females. However, this specialization may come at a price; increased competition for resources and greater risk of local extinction. The present study examined the effects of intraspecific colonization, carcass age, and exposure time on the colonization and oviposition responses of a specialist, primary colonizer, the secondary screwworm, *Cochliomyia macellaria*. Carcass age, exposure time, and colonization status (presence or absence of intraspecific colonization) significantly influenced the ovipositional response of *C. macellaria*. This species exhibited an oviposition preference for aged carcasses, with a tendency for higher oviposition after 8 h exposure time, but no preference between previously colonized or uncolonized carcasses. Mean egg hatch rate was also shown to be influenced by the aforementioned factors, with mean hatch rates varying between

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81.3 and 90.1% across various treatments. These results provide insight into mechanisms driving succession on carrion, as well as highlight the variation observed in successional studies for targeted species. Investigators relying on arthropod succession to indicate a time of colonization should proceed with caution in relying solely on the assumption primary colonizers only arrive and lay eggs, or larvae, on fresh carrion.

3.1 Introduction

Blow flies (Diptera: Calliphoridae) play an essential role in nutrient cycling of vertebrate carrion (Parmenter and MacMahon 2009, Payne 1965). The consumption of carrion allows for the breakdown of organic tissue and facilitates food web formation, community assembly and energy flow (Tomberlin *et al.* 2011). While the role of blow flies in these processes has been thoroughly studied, their role in ecosystem function on a larger scale remains understudied (Mohr and Tomberlin 2014). Consequently, the drivers of blow fly colonization of carrion and the role of this colonization pattern in carrion succession (i.e. the predictable appearance of carrion-associated arthropods) has historically been overlooked.

After environmental exposure, blow flies are able to locate remains within minutes, rapidly evaluating, ovipositing on, and feeding on the resource (Anderson and VanLaerhoven 1996). Being primary colonizers of carrion, there exists the potential for feeding larvae to face a high degree of intra- and interspecific competition (Reis *et al.* 1999, Shiao and Yeh 2008, Von Zuben *et al.* 2001), as well as predation by other organisms (Andrade *et al.* 2002, Faria and Godoy 2001). The presence of other organisms on carrion may also affect the timing and arrival of other primary and secondary colonizers (Brundage *et al.* 2014), as well as affecting offspring survival and fitness of those successional colonizers (Brundage *et al.* 2014).

A vast majority of the Calliphoridae colonizing carrion are limited in their feeding breadth, relying heavily on vertebrate remains for growth and development. However, some species, such as *Lucilia cuprina* (Wiedemann) (Diptera: Calliphoridae) are able to utilize multiple resources for nutrition, including feces/manure (Cook 1991). While dietary specialization may offer several advantages, such as a stronger interspecific competitive ability and higher optimal performance (Pulliam 1974), specialist foragers also face an increased risk of localized extinction in the absence of the preferred resource, and a higher degree of intraspecific competition (Pulliam 1974). The dietary breadth of an organism should always be considered on a specific scale, be it the carcass, the immediate environment, or a spatial or temporal scale. Arthropods feeding on vertebrate remains may represent generalist feeders, as they are able to consume multiple organs and tissues. However, when regarded on a landscape scale, the reliance on carrion for development may represent a specialized niche, thus deeming carrion-colonizing arthropods specialist foragers. Specialization on a specific resource may not only be limited to the type of resource; age specialization can also occur whereby certain species only colonize during a certain stage of decomposition (Goddard and Lago 1985, Lane 1975).

Oviposition on carrion by blow flies may depend on a number of factors. For example, the presence of other insects (Galindo *et al.* 2016), nocturnal/diurnal behavior patterns (Williams *et al.* 2017), temperature (Hans *et al.* 2018), moisture content (Browne 1962), and location/accessibility (Gunn and Bird 2011) have all been determined to influence oviposition behavior. Some species, such as *L. cuprina,* will only oviposit in regions where their tarsi are in contact with moisture (Browne 1962). Some species are more likely to oviposit or avoid oviposition when either conspecific or heterospecific eggs are already present (Brundage *et al.* 2017). Timing of oviposition may also be important. While it was previously noted that blow flies do not exhibit nocturnal oviposition, recent studies have found nocturnal oviposition for numerous species, including *Calliphora vicina* Robineau-Desvoidy, *Phormia regina* (Meigen), *Lucilia sericata* (Meigen), and *L. cuprina* (Berg and Benbow 2013, Greenberg 1990, Singh and Bharti 2001, Williams *et al.* 2017).

Numerous studies and case reports have highlighted the use of blow flies as time of colonization (TOC) estimators in medicolegal investigations (Babu *et al.* 2013, Bonacci *et al.* 2009, Turchetto *et al.* 2001). Various models have historically been used to estimate blow fly age, including egg aging through differential gene expression (Bourel *et al.* 2003, Tarone *et al.* 2007), larval length (Adams and Hall 2003, Byrd and Butler 1997), larval width (Day and Wallman 2006), pupal aging through cuticular analysis (Brown *et al.* 2015), and accumulated degree day models (Defilippo *et al.* 2013, Nabity *et al.* 2006). While each of these methods have been developed and shown to represent a reasonable degree of accuracy in aging individuals to determine TOC, few studies have accounted for the species interactions on carrion and how these may affect growth and development, and subsequent TOC estimates. Recently, focus has shifted to understanding successional patterns and how the interactions of various organisms on a carrion resource can shape the community structure and thus influence the TOC estimate (Matuszewski *et al.* 2010, Pechal *et al.* 2014, Wang *et al.* 2008). The present study aimed to evaluate the colonization and oviposition responses of a primary colonizer, *Cochliomyia macellaria* (Fabricius) 1) to variably aged carrion, 2) both in the presence and absence of a conspecific colonizer, and 3) when provided variable time for response.

3.2 Methods

3.2.1 *Cochliomyia macellaria* colony maintenance and egg acquisition

Cochliomyia macellaria was maintained at the Forensic Laboratory for Investigative Entomological Sciences (F.L.I.E.S) Facility, Texas A&M University, College Station, TX, and has been maintained for >30 generations, with wild-type individuals introduced periodically. Adults were maintained in standard insect rearing cages (BugDorm$^{©}$; 30 x 30 x 30 cm) at 27°C, a 14:10 L:D cycle and 60% RH. Cages were supplied with a mason jar containing water, along with a 1:1 honey and water solution and a 1:1 powdered milk and sugar mixture. To stimulate oviposition, adult flies were provided with a bovine blood meal (± 10 ml) five and seven days after emergence. Nine days after emergence, adults were supplied with ± 50 g bovine liver placed on a paper towel in a 90 ml plastic cup for a period of 8 h. Eggs were separated from liver and placed in a mason jar with vermiculite for rearing. After hatching, larvae were provided with bovine liver *ad libitum* until pupation. Upon emergence, adults were sexed and transferred in an equal sex ratio (250:250 M:F) to large insect rearing cages (84 x 84 x 132 cm; Chestnut Tools, Ontario, Canada) supplied with mason jars containing water, a 1:1 powdered milk and sugar mixture, and a 1:1 water and honey solution, and provided with a bovine blood meal at the same time points as described previously, until commencement, 9 d after emergence. *Cochliomyia macellaria* voucher specimens were submitted to the TAMU Insect collection under voucher number 742.

3.2.2 Treatment preparation

Uncolonized carcasses: For aged carcasses, rats (*Rattus rattus* Linnaeus; RodentPro$^{®}$; size Large [175-275 g]) were placed individually in sealed organza parcels (20 x 12 cm) in plastic storage containers (25 x 12 x 12 cm) and covered with plastic lids with cutouts (5 x 10 cm) covered with organza, in a standard fume hood (24°C, 60% RH and natural light cycle) five days prior to commencement of the trials. Fresh carcasses were removed from a freezer and thawed for 12 h at room temperature before trial commencement.

Colonized carcasses: Aged carcasses were prepared in the same manner as described above. Each carcass was inoculated with 0.3 g newly oviposited (<8 h) *C. macellaria* eggs on the first day of placement in the fume hood prior to sealing in the organza parcel. Larvae were left to hatch and consume the carcass undisturbed until commencement. The fresh carcasses were prepared in the same manner as described above, inoculated with 0.3 g *C. macellaria* eggs an hour before commencement, and the organza parcel resealed.

3.2.3 Experimental design

Oviposition assays: At commencement, each insect-rearing cage containing adult *C. macellaria* was supplied with a combination of treatments: (1) Uncolonized: one fresh uncolonized and one aged uncolonized rat carcass, and one empty control; (2) Colonized: one fresh colonized and one aged colonized (2nd/3rd instar, based on developmental stadium after inoculation) rat carcass, and an empty control; and (3) Delayed Colonization: one fresh colonized and one aged colonized (egg; inoculated on day of commencement) rat carcass, and one empty control. Adults were left undisturbed for 8 h in natural light conditions, 65% RH and 27°C to oviposit. After an 8 h exposure period, all rats were examined for fecal deposits on the organza covering. Any oviposited eggs were removed and weighed. Rats were returned to cages and left for a further 16 h and then reassessed. All oviposited eggs were stored in mason jars sealed with a WypeAll® covering, and allowed to hatch. Hatched larvae were preserved in 80% ethanol 48 h after conclusion of the experiment.

3.2.4 Statistical analyses

All statistical analyses were performed in R® (v3.5.2; 2018). As data met parametric assumptions, a two-way analysis of variance (ANOVA) was conducted for mean egg mass and mean hatch, with post-hoc tests using Tukey's HSD ($p < 0.05$).

3.3 Results

3.3.1 Oviposition preference

There were significant effects of exposure time ($F_{1,96} = 11.907$; $p < 0.05$) and carcass age ($F_{1,96} = 106.023$; $p < 0.05$) on the mass of eggs oviposited within each treatment group. There were also significant interactive effects of exposure time and carcass age ($F_{1,96} = 3.980$; $p < 0.05$) (Fig. 3.1), and carcass age and colonization status ($F_{2,96} = 7.341$; $p < 0.05$) (Fig. 3.2) on the mass of eggs deposited within each treatment group. There was no significant two-way interaction between exposure time and colonization status ($p > 0.05$) (Suppl. Fig. A3.1), and no significant three-way interaction between carcass age, colonization status, and exposure time was observed ($p > 0.05$) (Suppl. Fig. A3.2). Across all treatments, adult *C. macellaria* exhibited a site preference for oviposition on aged carcasses, regardless of colonization status or exposure time. The greatest mean mass of eggs deposited was observed in the delayed colonization group, on the aged carcass after an exposure period of 8h, with a mean mass of 0.574 ± 0.247 g. Across all treatment groups, the mass of eggs deposited was greater after an exposure time of 8 h than after the 24 h exposure time, regardless of colonization status.

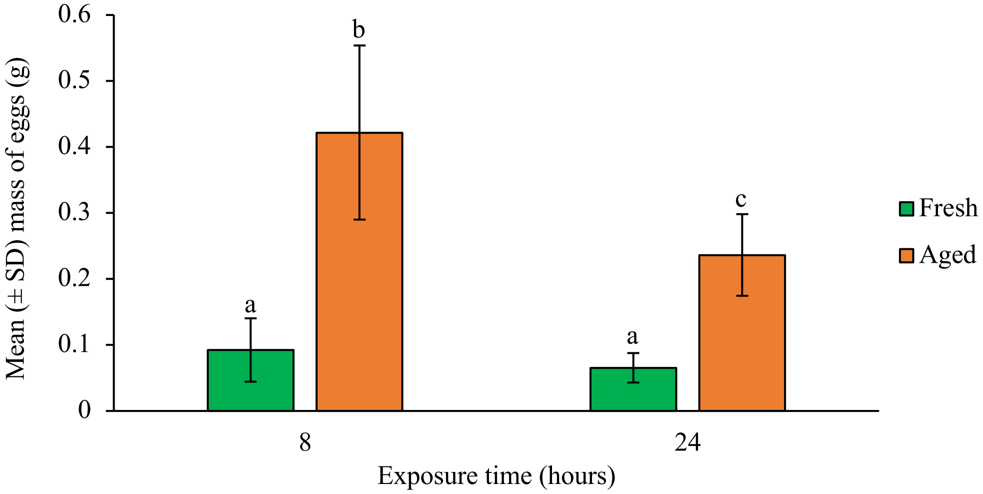

Figure 3.1: Effect of carcass age and exposure time on the mean (± SD) mass of eggs (grams) oviposited by *C. macellaria* females held at 27°C and 65% RH. Shared letters indicate no significant differences.

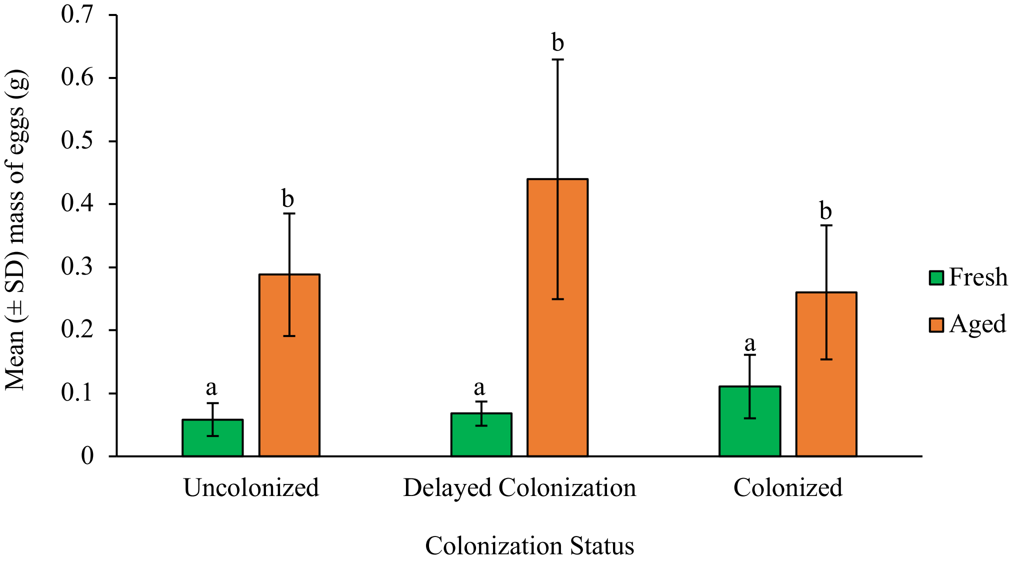

Figure 3.2: Effect of carcass age and colonization status on the mean (± SD) mass of eggs (grams) oviposited by *C. macellaria* females held at 27°C and 65% RH. Shared letters indicate no significant differences.

3.3.2 Egg hatch

There were significant effects of carcass age ($F_{1,96} = 35.573$; $p < 0.05$) and colonization status ($F_{2,96} = 4.739$; $p < 0.05$) on the mean hatch rate of oviposited eggs across treatments. There was also a significant interactive effect of carcass age and colonization status ($F_{2,96} = 4.608$; $p < 0.05$) on the mean hatch rate of oviposited eggs (Fig. 3.3). There were no significant interactive effects of colonization status and exposure time ($p > 0.05$) (Suppl. Fig. A3.3) or carcass age and exposure time ($p > 0.05$) (Suppl. Fig. A3.4), nor a significant three-way interaction of carcass age, exposure time, and colonization status ($p > 0.05$) (Suppl. Fig. A3.5) on the mean hatch rate of oviposited eggs. Across all treatments, there was an overall slightly higher hatch rate on aged carcasses than on fresh carcasses. Mean hatch rates across treatments ranged between 81.3% and 91.0%.

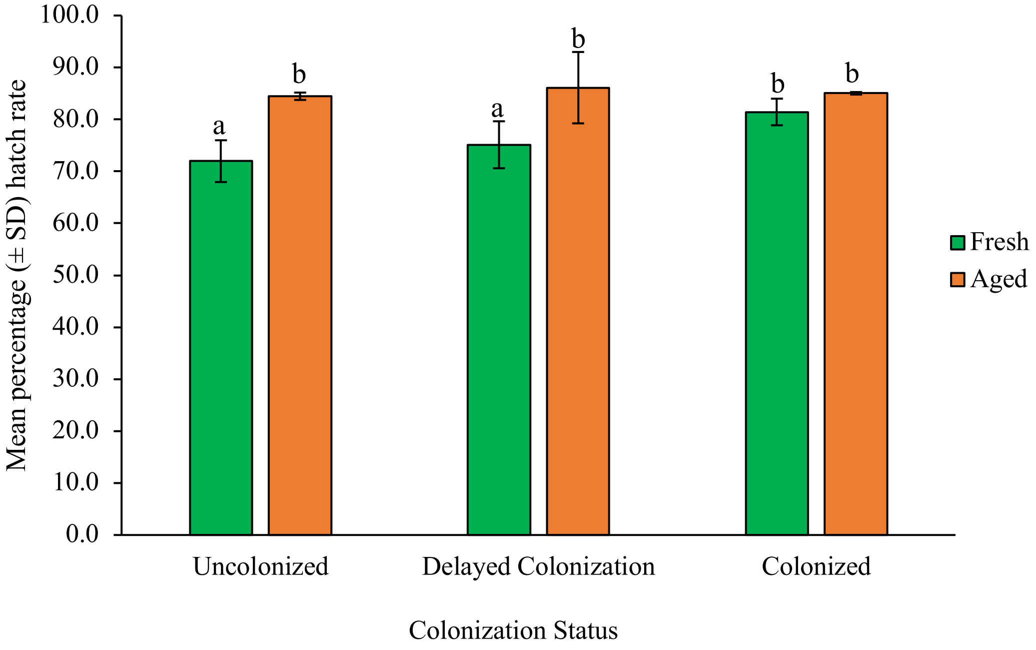

Figure 3.3: Effect of colonization status and carcass age on the mean percentage (± SD) of eggs hatching from eggs oviposited by *C. macellaria* females held at 27°C and 65% RH. Shared letters indicate no significant differences.

3.4 Discussion

Based on our survey of the literature, this study is the first to investigate the variation in colonization time by a species previously assumed to be solely a primary colonizer (Hall and Doisy 1993, James 1947). This assumption, however, may be regionally specific and may be influenced by seasonality and/or temperature as well (Benbow *et al.* 2013). In addition to examining oviposition site preference at variably aged resources, the present authors also investigated variation in hatch rate and examined visitation to carcasses of different ages. *Cochliomyia macellaria* exhibited a tendency to oviposit at aged carcasses in larger numbers than at fresh carcasses, across all treatments, regardless of colonization status or exposure time.

These data provide valuable insight into the highly plastic nature of *C. macellaria,* further lending support to the flexible nature of this species in other aspects of its biology, such as its responses to varying temperature regimes and substrate types (Boatright and Tomberlin 2010, Owings *et al.* 2014). A delay in colonization by *C. macellaria* of up to 24 h after environmental exposure has also been reported, with adult numbers peaking 48 – 96 h after exposure (Hall and Doisy 1993). The variability in colonization time has also been noted in other Calliphoridae species, with *Phormia regina* (Meigen) and *Lucilia illustris* (Meigen) adults present at carcasses during fresh, bloat, active, advanced, and dry remains stages of decomposition, a period of ~271 days (Anderson and VanLaerhoven 1996, Pechal *et al.* 2014, Schoenly and Reid 1987). Additionally, Arnaldos *et al.* (2004) found both *Calliphora vicina* and *Chrysomya albiceps* (Wiedemann) present during the fresh and active decay stages, and *Lucilia sericata* active and present through the fresh, active and advanced decay stages (Arnaldos *et al.* 2004).

Cochliomyia macellaria has been assumed to be a primary colonizer (Tomberlin and Adler 1998). However, data from the current study demonstrate greater plasticity in terms of colonization preference by this species. These data draw attention to the assumption that stages of decomposition have predictable arthropod community structure; this assumption is not true in all cases. Schoenly and Reid (1987) evaluated the timing and arrival of arthropods at a carrion resource, describing the stages of decomposition, and the subsequent arthropod arrival, as a continuum rather than discrete stages, with arthropod arrival and departure subject to fluctuation. The data from the present study support the view that arthropod arrival and departure is not discrete; specifically, *C. macellaria*, can be present, and actively colonizing remains, during numerous stages of the decomposition process.

Carcass age has been shown to significantly influence the community structure of arthropods colonizing carrion (McKinnerney 1978). Diversity in carrion-colonizing Diptera peaks around three days post-exposure (Cruise *et al.* 2018), with this increased diversity of species generally lasting through the bloat stage, 2 – 8 d, depending on temperature (Anderson and VanLaerhoven 1996, McKinnerney 1978). The increased attraction to carrion with time, and thus carcass age, by a variety of species may span a period of carcass assessment prior to colonization. Anderson and VanLaerhoven (1996) found adult *P. regina* visiting hen carcasses within 24 h of outdoor placement, but no oviposition occurred during this time. Oviposition only began between 24 and 48 h after placement, and peaked between 48 and 72 h after placement (Anderson and VanLaerhoven 1996, Weatherbee *et al.* 2017), indicating a period where adults are attracted to the carcass, and potentially assessing the remains for suitability, before oviposition occurs when the carcass has aged somewhat. While the results of the aforementioned study are not directly comparable to the present study, the inclination of an increased oviposition response to an aged carcass over a fresh carcass reinforce the preference of attraction to carcasses that have been exposed for a period of time as found in other studies. In this study, carcass age also significantly affected the mean egg hatch, potentially due to an increased ease of digestibility for hatching larvae on an aged carcass (Shukla *et al.* 2018). Regardless of treatment, the mean egg hatch rate was comparable to the standard hatch rate observed for other studies utilizing *C. macellaria* (Chaudhury and Skoda 2013).

The presence of both conspecific and heterospecific larvae has been shown to significantly affect arrival patterns of carrion-colonizing arthropods (Weatherbee *et al.* 2017a). Heterospecific colonization may indicate potential competition and/or predation, whereas conspecific colonization, in this case, presents only the potential for competition. However, within eusocial insects such as honey bees and ants, relatedness often decreases the intraspecific competition between individuals (Reeve and Hölldobler 2007). Picard and Wells (2014) noted a high degree of relatedness between adult *P. regina* attending carcasses in a natural population, citing that a female may lay hundreds of eggs at a carcass, resulting in emerging larvae being full siblings. The population utilized for the present study had been reared in a laboratory for >30 generations, and a high degree of relatedness among individuals was likely. Based on these findings in other species, the presence of closely-related conspecific larvae is unlikely to have significantly affected the site choice by ovipositing females, since colonization status was not a significant factor in site choice. The presence of conspecific feeding larvae significantly affected the mean hatch rate of eggs oviposited at colonized treatments. While the actively feeding larvae were not in direct contact with oviposited eggs, towards the end of the exposure period, a small number ($n < 10$) had escaped the mesh coverings and were moving freely in the plastic containers housing the rats. It is highly possible that these larvae could have disturbed the egg clutches that had been deposited, slightly decreasing the mean egg hatch on the aged carcass in the colonized treatment and leading to the observation of no significant difference in hatch rate between the fresh and aged carcasses in the colonized treatments.

While this study is the first of its kind to evaluate the colonization preference of adult *C. macellaria* on variably aged carcasses, the colony used in this study has been reared in a laboratory for >30 generations, and there exists the potential that this response may be population specific. Although the laboratory population has been reared on previously frozen, thawed bovine liver which has not aged or decomposed significantly, the freezing may influence the microbiome of the substrate (Flores *et al.* 2015) and thus influence the chemical attractiveness of ovipositing adults based on the substrate provided for larval rearing. Closely related to the microbiome of the liver is the comparison between the homogenous liver that larvae are fed, and the heterogenous substrate provided by a decaying rat. The microbiome on a single homogenous substrate, regardless of whether it has been previously frozen or not, is vastly different from the microbiome on a heterogenous substrate, such as a complete organism like a rat (Li *et al.* 2017).

To accurately assess the preference to all treatments explored in the current study, future research should allow sexually mature flies to choose between both fresh and aged, colonized and uncolonized rat carcasses simultaneously. The results from such a study will likely clarify the exact role of conspecific colonizers and carcass age on the true colonization preference of this species. In addition, studies such as the one presented here should be repeated on a greater variety of species, particularly those species that are deemed primary colonizers and are commonly used for time of colonization (TOC) estimates in medicolegal investigations. A further investigation should also evaluate the effects of colonization when rat carcasses are inoculated and colonized with heterospecific larvae, both potential competitors, as well as potential predators. While the priority colonization effects of *C. macellaria* and *Chrysomya rufifacies* Macquart have been investigated

(Brundage *et al.* 2014), the aforementioned study provided larvae with fresh bovine liver only, without evaluating the effects of carcass age or heterogenous substrate on this relationship.

The findings of the present research highlight the potential of certain blow fly species to be active as primary or secondary colonizers, and bring into reconsideration the utility and accuracy of these species as time of colonization indicators in criminal investigations. The reliance on ecological succession on carrion as an indicator of time of colonization should also be reported with caution, noting the variation in succession by arthropods and considering potential drivers of this variation in relation to criminal investigations.

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CHAPTER IV

INFLUENCE OF SUBSTRATE AGE AND INTERSPECIFIC COLONIZATION ON OVIPOSITION BEHAVIOR OF A GENERALIST FEEDER, BLACK SOLDIER FLY, *HERMETIA ILLUCENS* L. (DIPTERA: STRATIOMYIDAE) ON CARRION RESOURCES

Arthropods colonizing carrion have developed numerous strategies to optimally develop in an ephemeral resource. Variation in feeding strategies plays a significant role in determining the success or failure of a species. Generalist species across numerous genera have exhibited success in this feeding strategy, largely due to decreased risk of resource depletion and competition. The present study investigated the colonization and oviposition habits of a generalist carrion feeder, *Hermetia illucens*, to varying conditions of carrion decay and colonization. Oviposition treatments consisted of varying combinations of fresh and 5-day old decomposing rats, uncolonized and colonized with larvae of a primary blow fly colonizer, *Cochliomyia macellaria.* The black soldier fly exhibited a 49.3% greater preference for oviposition at aged resources, regardless of carcass colonization status. However, with the introduction of a plant-based diet, there was a significantly higher (40.4%) oviposition response to this diet. Results of this study highlight the potential inaccuracies in the utility of *H. illucens* as time of colonization indicator species for legal investigations, and propose that black soldier fly larvae might be considered as exploitative niche-specialists when found in association with carrion, rather than a generalist feeder species on carrion.

4.1 Introduction

Carrion resources represent discrete, patchy, ephemeral resources utilized by arthropods, vertebrate scavengers, and microbes for growth and survival (Barton *et al.* 2013). While some carrion-feeding arthropod species are heavily reliant on carrion for nutrients, some necrophagous arthropods exhibit a wider feeding breadth and are not solely reliant on carrion (Mohr 2012) Commonly defined as specialist and generalist feeders respectively, arthropods engaging in each strategy have developed unique and specific life-history and physiological traits to optimally utilize carrion (Mohr and Tomberlin 2015). Species with a wider dietary breadth, or generalist feeders, are able to utilize a multitude of resources to meet their nutritional requirements. This strategy offers a number of advantages, the most prominent of which is a decreased risk of complete resource depletion; if one preferred resource becomes unavailable, numerous other resources are still available for growth and survival (Pulliam 1974, Pyke *et al.* 1977). However, with such a strategy comes limitations, including reduced optimal performance, and increased metabolic costs of processing a wider range of compounds ingested through feeding (Barrett and Heil 2012). Regardless, the distinction between feeding habits may not extend to habitat preferences; specialist feeders may be generalists with regard to habitat choice, while generalist feeders may exhibit habitat specialization (Kočárek 2003). Feeding preference may also refer to the age of carrion preferred by arthropods; some species are specialists for fresh carrion, while others will oviposit and develop on older carrion resources (Mohr 2012).

Blow flies (Diptera: Calliphoridae) have been thoroughly investigated as carrion colonizers. Various biological aspects, ranging from physiological (Boatright and Tomberlin 2010, Byrd and Butler 1996, 1997, Kotzé *et al.* 2016, Owings *et al.* 2014), ecological (Benbow *et al.* 2015, van der Niet *et al.* 2011), behavioral (Boulay *et al.* 2015, 2016, Liu *et al.* 2016), and genetics

(Bharti and Singh 2017, Concha *et al.* 2012, McKenzie *et al.* 1982, Warman *et al.* 2000), have been explored, and each of these has been placed in the context of estimating a time of colonization (TOC) in legal investigations. Numerous species interactions involving blow flies have also been investigated, including competition (Denno and Cothran 1976, Smith and Wall 1997), predation (Faria and Godoy 2001, Omar 1995) and spatial segregation (Pimsler *et al.* 2019).

Blow flies associated with carrion are, for the most part, specialist feeders, solely reliant on carrion for growth, reproduction, and survival (Smith and Wall 1997). Calliphorid species that frequent carrion exhibit a variety of colonization strategies, including variation in colonization time (Moophayak *et al.* 2017), colonization avoidance or preference in the presence of conspecifics and heterospecifics (Brundage *et al.* 2017), and the ability to become facultative predators (Cammack and Nelder 2010). A vast majority of blow flies are primary colonizers, arriving at remains within minutes after environmental exposure, and rapidly colonizing the resource (Tomberlin and Adler 1998). The initial colonization of remains by primary colonizers may deter other arthropods from colonizing both during early and later stages of decomposition due to resource depletion and potential competition (Brundage *et al.* 2017, Weatherbee *et al.* 2017).

In contrast to blow flies associated with carrion, the black soldier fly, *Hermetia illucens* (L.) (Diptera: Stratiomyidae) is considered a detritivore as it able to colonize a variety of resources including carrion. This species has been documented feeding as larvae on manure, plant waste, and even restaurant/food waste (Diener *et al.* 2011, Wang and Shelomi 2017). This species overlaps with many green bottle blow fly species globally, as it thrives in warmer temperatures at high humidity (Sheppard *et al.* 1994, Tomberlin and Sheppard 2002). Adults do not need to feed, and larvae exhibit an extended larval duration to assimilate sufficient nutrients to survive through pupation and adulthood (Sheppard *et al.* 2002). Gravid adults will oviposit in crevices close to a resource, with larvae emerging after approximately four days (Booth and Sheppard 1984, Stephens 1974). Historically, a majority of the research surrounding this species has been focused on its efficiency for waste bioconversion (Li *et al.* 2011, Paz *et al.* 2015, Salomone *et al.* 2017, Surendra *et al.* 2016), and on mass rearing as an alternative food source (Bondari and Sheppard 1987, Newton *et al.* 1977, Wang and Shelomi 2017). However, *H. illucens* has been documented to occur on human remains and offers potential as an indicator of time of colonization (TOC) in medicolegal investigations (Carvalho *et al.* 2000, Lord *et al.* 1994). The timing of their arrival to a carcass, however, remains in question. While some studies report arrival during early decomposition (Tomberlin *et al.* 2005), others report arrival patterns during the later stages of decomposition (Dunn 1916, Lord *et al.* 1994, Martínez-Sánchez *et al.* 2011). Harnden and Tomberlin (2016) showed that rearing *H. illucens* larvae exclusively on pork or beef tissue significantly extended the larval period, and a field validation of laboratory obtained data showed inconsistencies in accurately estimating larval age for this species. The timing and arrival and colonization of black soldier fly to vertebrate remains may influence the TOC estimation by entomologists, hence it is of utmost importance to determine the attraction and colonization responses of this species to carrion, and thus clarify the accuracy and precision of estimates based on colonization by this species.

The present study aimed to evaluate the colonization and oviposition preferences of the black soldier fly, *H. illucens*, at variably aged carrion resources, and establish differences in preferences based on the presence of a primary carrion colonizer, *Cochliomyia macellaria* (Fabricius) (Diptera: Calliphoridae), a species commonly encountered in the southern USA where the black soldier fly also occurs.

4.2 Methods

4.2.1 *Cochliomyia macellaria* colony maintenance and egg acquisition

Cochliomyia macellaria was maintained at the Forensic Laboratory for Investigative Entomological Sciences (F.L.I.E.S) Facility, Texas A&M University, College Station, TX, USA and has been maintained for >30 generations, with wild-type individuals introduced periodically. Adults were maintained in standard insect rearing cages (BugDorm©; 30 x 30 x 30 cm) at 27°C, a 14:10 L:D cycle and 60% RH. To stimulate oviposition, adult flies were provided with a bovine blood meal (± 10 ml) five and seven days after emergence. Nine days after emergence, adults were supplied with ± 50 g bovine liver placed on a paper towel in a 90 ml plastic cup for a period of 8 h. Eggs were separated from liver and placed in a mason jar with vermiculite for rearing. After hatching, larvae were provided with bovine liver *ad libitum* until pupation. Upon emergence, adults were transferred to BugDorm© cages.

4.2.2 *Hermetia illucens* colony maintenance & adult acquisition

Black soldier fly eggs were obtained from a colony housed at the F.L.I.E.S Facility at Texas A&M University, College Station, TX, USA, which originated from the Coastal Plain Experiment Station in Tifton, GA, USA. The colony in use has been maintained for >100 generations. To stimulate oviposition, cardboard stacks (10 x 5 cm x 5 layers; 0.5 cm flutes) were placed in the cage for eight hours during normal daylight hours. Eggs were weighed after oviposition and separated into 1 g batches. After four days, hatched larvae were transferred to 40 g standard Gainesville diet, and incubated at 27°C, 70% RH and 14:10 L:D cycle. Larvae were reared by incremental feeding procedures every second day, using mass increments of 40 g, 400 g and 1 kg until pupation. Pupae were separated from spent media and allowed to emerge in the same incubation conditions. Upon emergence, adults were sexed and transferred in an equal sex ratio (250:250 M:F) to insect rearing cages (84 x 84 x 132 cm; Chestnut Tools, Ontario, Canada) supplied with mason jars containing water.

4.2.3 Treatment preparation

Uncolonized carcasses: For aged carcasses, rats (RodentPro®; size Large [175-275 g]) were placed individually in plastic storage containers (25 x 12 x 12 cm) and covered with plastic lids with cutouts (5 x 10 cm) covered with mesh, in a standard fume hood (24°C, 60% RH and natural light cycle) five days prior to commencement. Fresh carcasses were removed from a freezer and thawed for 12 h at room temperature before commencement.

Colonized carcasses: Aged carcasses were prepared in the same manner as described above. Each carcass was inoculated with 0.3 g newly oviposited (<8 h) *C. macellaria* eggs on the first day of placement in the fume hood. Larvae were left to hatch and consume the carcass undisturbed until commencement. The fresh carcasses were prepared in the same manner as described above, and inoculated with 0.3 g *C. macellaria* eggs an hour before commencement.

Gainesville diet: Approximately, 250 g Gainesville diet, which is a standard for rearing black soldier flies (Sheppard *et al.* 2002), was prepared on the morning of the experiment start. This diet was placed in the same sized containers as used for rat treatments, with the same mesh-insert lid.

4.2.4 Experiment design

At commencement, each insect-rearing cage containing adult *H. illucens* was supplied with a combination of treatment; (i) one fresh uncolonized and one aged uncolonized rat carcass, and one empty control; (ii) one fresh uncolonized and one aged uncolonized rat carcass, a standard

Gainesville diet, and an empty control; (iii) one fresh colonized and one aged colonized rat carcass, and one empty control; and (iv) one fresh colonized and one aged colonized rat carcass, a standard Gainesville diet, and an empty control. A cardboard stack (known weight; 4 layers, 5 x 5 cm) was placed over the mesh insert of each container lid. Adults were left undisturbed for 8 h in natural light conditions, 65% RH and 27°C to oviposit. After an 8 h exposure period, all egg stacks were removed from treatments. Cardboard stacks were weighed to determine egg mass oviposited, and eggs incubated in a mason jar sealed with a breathable WipeAll® towel until emergence. Five days after oviposition, emerged larvae were euthanized in ethanol and counted. *Cochliomyia macellaria* and *H. illucens* voucher specimens were submitted to the TAMU Insect Collection under voucher number 742.

4.2.5 Statistical analyses

All statistical analyses were performed in R® (v3.5.2 – "Eggshell Igloo"; 2018), using package *lme4* (Bates *et al.* 2015). Generalized linear model (GLM) analyses were performed to evaluate differences between treatments, with a follow up of pairwise comparisons where significant differences were observed ($p < 0.05$). The model was constructed with a Gamma distribution to account for data that were not normally distributed.

4.3 Results

4.3.1 Choice study I: Fresh rat vs aged rat

All oviposition activity: There was a significant effect of carcass age on the mean mass of eggs oviposited, when considering all available options, with a 49.3% greater preference for oviposition at the aged carcass ($t_{1,35} = -2.881$; $p < 0.05$) (Fig. 4.1). There was no effect of colonization status on mean egg mass of eggs oviposited (Suppl. Fig. A4.1).

Egg masses excluding egg-free carcasses: When considering egg masses only from rats that showed oviposition activity, there was a significant effect of carcass age on the mean mass of eggs oviposited ($t_{1,26} = -2.860$; $p < 0.05$) (Fig. 4.2), with a 33.87% greater oviposition preference to oviposition on the aged carcass. There was no significant effect of colonization status on the mean mass of eggs oviposited (Suppl. Fig. A4.2).

Egg hatch: There was a significant interaction of carcass age and colonization status on the mean percentage of eggs hatching at each of the rat carcasses where eggs were oviposited ($t_{3,26} = 2.377$; $p < 0.05$) (Fig. 4.3), with the greatest mean percentage hatch (87.9%) on fresh, uncolonized rat carcasses.

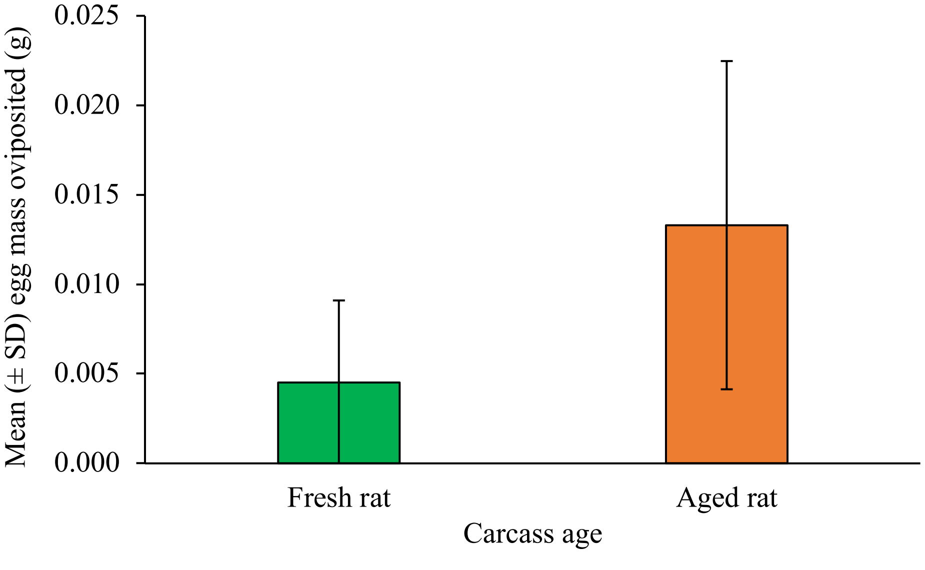

Figure 4.1: Mean (± SD) mass of eggs oviposited by *H. illucens* adults at fresh and aged decomposing rat carcasses after an exposure time of 8 h, taken as a function of all treatments, including those where no oviposition activity was recorded. Figure represents untransformed data.

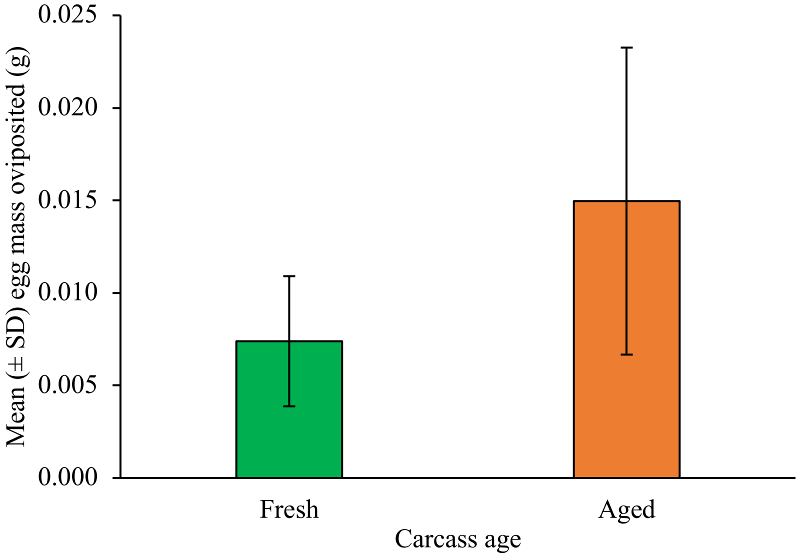

Figure 4.2: Mean (± SD) mass of eggs oviposited by *H. illucens* adults at fresh and aged decomposing rat carcasses after an exposure time of 8 h, taken as a function of treatments only where oviposition activity was recorded. Figure represents untransformed data.

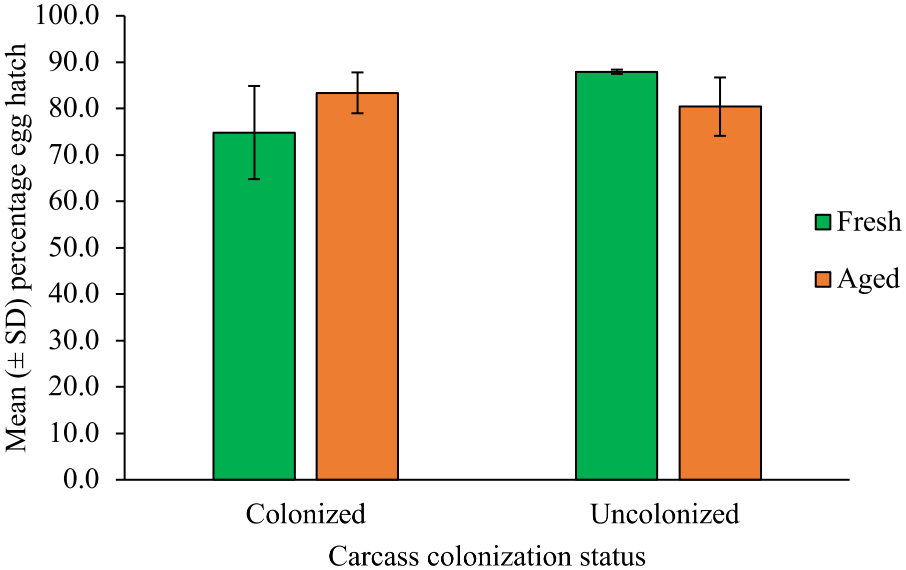

Figure 4.3: Mean (± SD) percentage egg hatch of eggs oviposited by *H. illucens* adults on combinations of fresh and aged rat carcasses, colonized with *C. macellaria* adults, and uncolonized carcasses, after an exposure period of 8 h for oviposition, and incubation period of 5 d for hatching. Figure represents untransformed data.

4.3.2 Choice study II: Fresh rat vs aged rat vs Gainesville diet

All oviposition activity: With the inclusion of a plant-based Gainesville diet, there was a significant effect of rat colonization status on the egg mass oviposited, with the consideration of all possible treatment options, showing significantly higher (39.5%) egg masses at the colonized carcasses ($t_{1,53} = -4.304$; $p < 0.05$) (Fig. 4.4). There was also a significant effect of substrate type on the mean mass of eggs oviposited ($t_{2,53} = 6.714$; $p < 0.05$), with significantly higher (40.4%) egg masses at the Gainesville diet than at either of the rat carcasses (Fig. 4.5). There was no significant interactive effect of colonization status and substrate type on the mean mass of eggs oviposited.

Egg masses excluding egg-free carcasses: When considering egg masses only from rats that showed oviposition activity, there was a significant effect of substrate type on the mean mass of eggs oviposited ($t_{2,37} = 5.828$; $p < 0.05$) (Fig. 4.6), with a 15.4% greater preference for oviposition on the Gainesville diet. There was no significant effect of colonization status on the mean mass of eggs oviposited (Suppl. Fig. A4.3).

Egg hatch: There was a significant effect of substrate type on the mean percentage of eggs hatching at each of the substrates where eggs were oviposited ($t_{2,37} = 2.985$; $p < 0.05$) (Fig. 4.7), with a significantly higher percentage (4%) hatch from eggs oviposited at the Gainesville diet. There was no significant effect of colonization status on the mean percentage egg hatch across treatments (Suppl. Fig. A4.4).

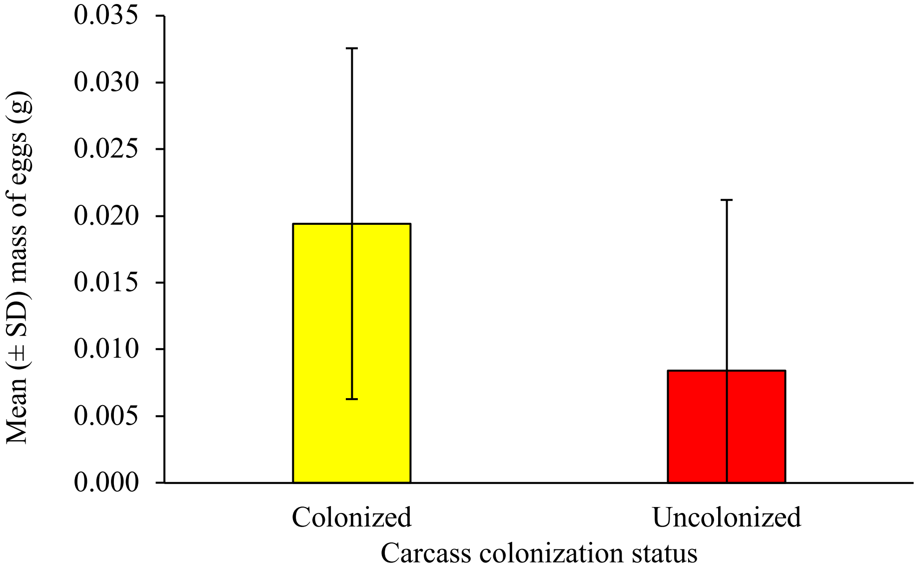

Figure 4.4: Mean (± SD) mass of eggs oviposited by *H. illucens* adults at rat carcasses colonized with a primary colonizer, *C. macellaria*, and uncolonized rat carcasses, after an exposure time of 8 h, taken as a function of all treatments, including those where no oviposition activity was recorded. Figure represents untransformed data.

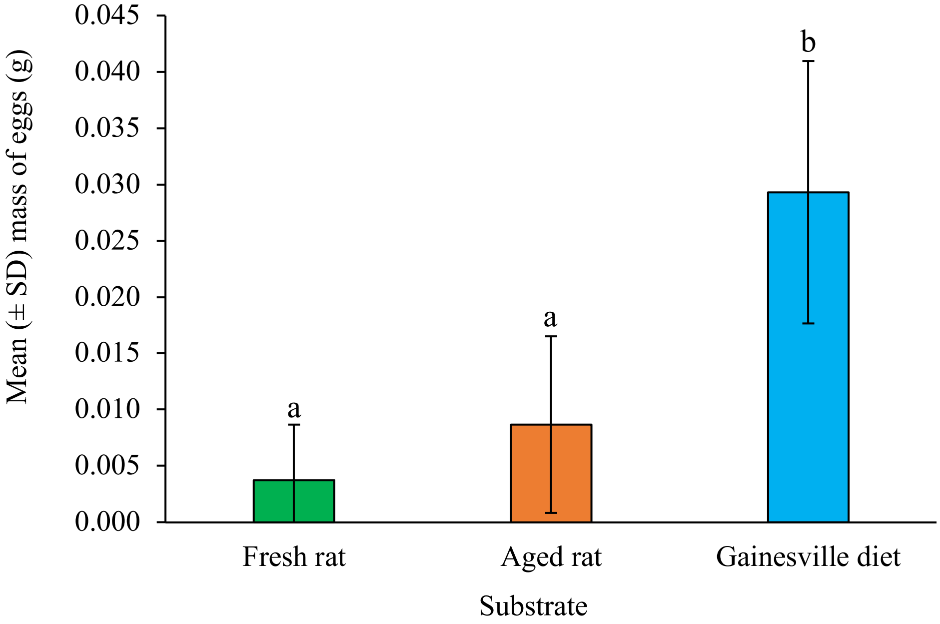

Figure 4.5: Mean (± SD) mass of eggs oviposited by *H. illucens* adults at fresh and aged decomposing rat carcasses, and Gainesville diet, after an exposure time of 8 h, taken as a function of all treatments, including those where no oviposition activity was recorded. Shared letters indicate no significant differences. Figure represents untransformed data.

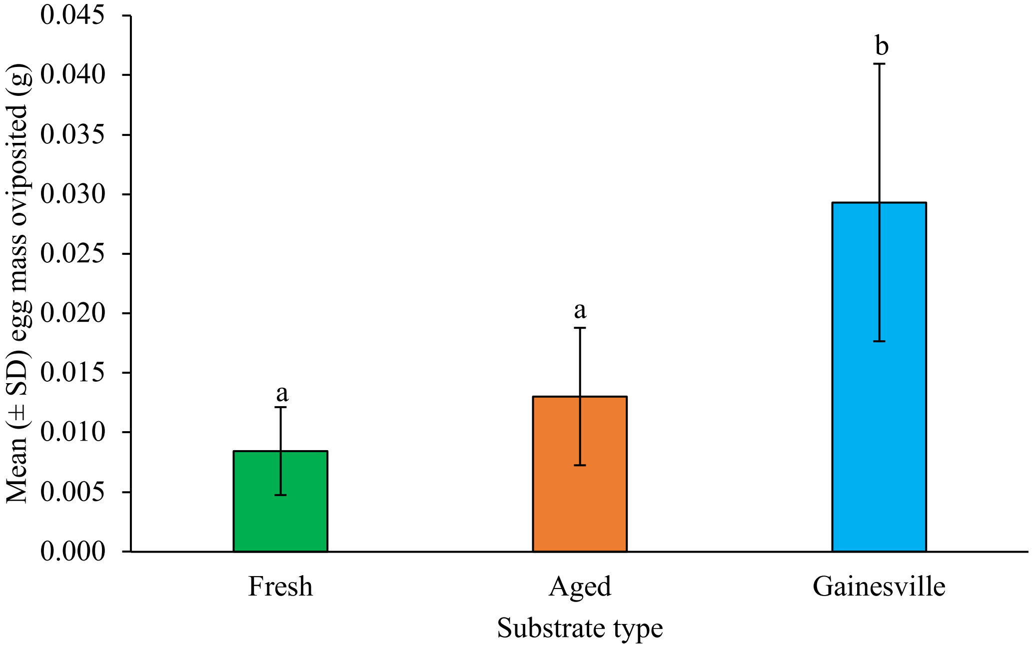

Figure 4.6: Mean (± SD) mass of eggs oviposited by *H. illucens* adults at fresh and aged decomposing rat carcasses, and a standard Gainesville diet, after an exposure time of 8 h, taken as a function of treatments only where oviposition activity was recorded. Shared letters indicate no significant differences. Figure represents untransformed data.

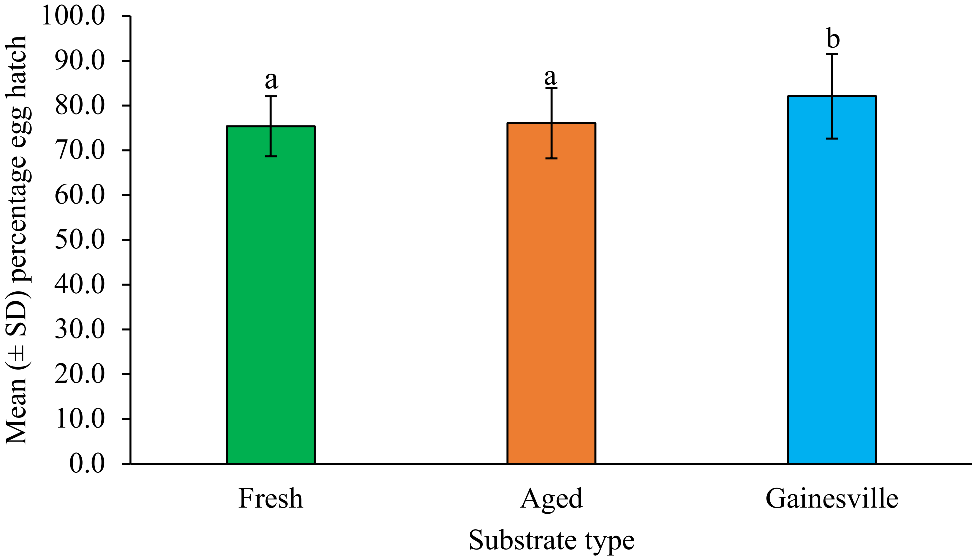

Figure 4.7: Mean (± SD) percentage egg hatch from clutches oviposited at various substrate types by *H. illucens* adults, after an exposure period of 8 h, and an incubation period of 5 d. Shared letters indicate no significant differences. Figure represents untransformed data.

4.4 Discussion

Based on an extensive literature review, this study is the first to evaluate the oviposition preferences on black soldier fly, *H. illucens*, on variably aged carrion resources, both in the presence and absence of a primary colonizer, *C. macellaria*. The results of this study reveal that the presence of a primary colonizer is not a driving factor when this species is selecting an oviposition site, and that there is a greater influence of substrate type in driving oviposition site selection. Given the option of only variably aged carrion resources, there is a preference of oviposition on an aged carcass. However, with the introduction of a plant-based substrate (Gainesville diet), there was a significantly higher preference for oviposition at the Gainesville site than at either of the carrion resources available. Greater egg masses were oviposited at aged carcasses when only decaying rats were provided, and when plant matter was provided, these yielded the greater egg masses. Mean percentages of egg hatch showed a high (>75%) mean hatch of eggs across all treatments, showing a high likelihood of fertile eggs being oviposited in all instances. Typical egg hatch of this population is around 85% (J. Cammack; pers. comm).

The higher frequency of oviposition at aged carcasses may be indicative that this species is reliant on the by-products produced through microbial metabolism and decomposition or the carcass (Paczkowski and Schütz 2011). Through the action of microbes and insects, complex constituents of the carcass, such as carbohydrates, are broken down into simpler substances (Barton *et al.* 2013). Furthermore, the excretions and secretions (ES) released during blow fly larval feeding has been shown to substantially increase digestibility of resources for other species consuming the carrion (Pinilla *et al.* 2013).

However, *H. illucens* exhibited oviposition behavior at both fresh and aged resources. The propensity to colonize both fresh and aged resources once again supports generalist feeding behavior in the larvae of this species (Diener *et al.* 2011, Wang and Shelomi 2017), exhibiting a broad feeding breadth with the ability to colonize, and potentially utilizing variably aged carrion (Dunn 1916, Lord *et al.* 1994, Pujol-Luz *et al.* 2008). Furthermore, *H. illucens* has been found associated with carrion, and has been used as an indicator of the post mortem interval (PMI) (Dunn 1916, Lord *et al.* 1994, Pujol-Luz *et al.* 2008, Tomberlin *et al.* 2005). In all instances where this species has been used to indicate PMI, it has been found in outdoor scenes, in close association with vegetation. In laboratory cultures, this species is commonly reared on a standard diet consisting of plant matter (Sheppard *et al.* 2002), where it has been shown to perform well, with a high survival through pupation. However, when reared exclusively on a homogenous animal-based diet (e.g., pork or beef), the larval stages of this species are significantly extended when compared to a grain-based diet (Harnden and Tomberlin 2016), with decreased survivorship. Based on these observations, and the results of this study, it is feasible to speculate that while adult soldier flies may be attracted to carrion (possibly based on volatile organic compound (VOC) emissions, many of which overlap with decaying plant matter), but when larvae begin to develop, they are unable to survive solely on the carrion resource due to the nutritional limitation for this species (Cammack and Tomberlin 2017). By migrating to the ecotone between the carcass and the ground, an area rich in decaying vegetation and carcass leachate, the larvae face little interspecific competition and can exist as fringe species. Fringe species at carrion are not unique to *H. illucens*; *Chrysomya rufifacies* (Macquart) (Diptera: Calliphoridae) have recently been reported as feeding at the same ecotone as *H. illucens* (Pimsler *et al*. 2019). However, *Ch. rufifacies* larvae are not known to feed on decaying vegetation, and are thus assumed to be exploiting this ecotone in a different way to *H. illucens*. The variation in feeding strategies between these two species in the specialized niche further supports the need for a functional approach to describing arthropod utilization of carrion, in addition to a structural approach.

The presence of the primary colonizer in this study elicited a significantly different response to the results obtained in the absence of a primary colonizer. These data suggest that the sequence of arthropod succession on carrion may be significantly altered if there is a delay in the arrival of primary colonizer species, or if primary colonizers are utilizing material in the carrion ecosystem, rather than the vertebrate tissue itself. By relying exclusively on assumed and predicted patterns of succession (Anderson and VanLaerhoven (1996), there exists the possibility of introducing errors in estimating the time of colonization (TOC) in criminal cases; thus, succession patterns should be meticulously examined before inferring TOC based on presence or absence of predicted species.

While this study is the first of its kind to delve into the mechanisms driving *H. illucens* attraction and colonization of vertebrate carrion, it must be considered that the population used in this study has been maintained for >100 generations on a grain-based diet without the introduction of any animal waste into the rearing system. Soldier fly oviposition has been observed in the College Station area at pig carcasses (T. Rusch; pers. comm.), but wild-type individuals have not been recently introduced into the colony. There exists the possibility that the results obtained from this study stem from a chemical legacy and predisposition to oviposition on a grain-based diet (Gainesville diet), and may not accurately reflect the oviposition site choices of a wild population.

Future work should aim to evaluate oviposition preferences in this species when exposed to a greater variety of aged resources, ranging through all the defined stages of decomposition (Payne 1965). This species has been most frequently associated with drier remains (Lord *et al.* 1994), but due to an extended larval life cycle, this may not be the stage at which oviposition occurs. Furthermore, microbial isolation from both animal and plant remains can be used as a tool to evaluate the changes in volatile organic compounds (VOCs) emitted by both types of remains (Pechal *et al.* 2013). In a simple Y-tube experimental setup, one could evaluate the drivers of attraction to carrion by adults of this species; the decomposing animal remains or decaying plant matter below the remains.

The results of this study bring into question the utility of *H. illucens* as indicator species in TOC estimates. Having extended life cycles, this species may be useful in indicating that remains have been exposed to the environment for a prolonged period of time. But due to their preference of ovipositing at aged remains rather than fresh, TOC estimates based on these species may not be precise or accurate. As niche-specialists, however, this species has evolved to exploit a niche that is infrequently studied in carrion ecosystems; a nutrient-rich, protected environment with low competition and high reward.

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CHAPTER V

SUMMARY

5.1 Rationale of research

Extensive research has been conducted into understanding the biological aspects of arthropod colonization and utilization of carrion. The biotic and abiotic factors affecting attraction and colonization of carrion resources have been extensively documented, and experimentally manipulated (Jordan and Tomberlin 2017). The data from such research have contributed to the wealth of knowledge available regarding the post-colonization interval; a period of insect activity on vertebrate remains (Tomberlin *et al.* 2011). However, the focus of understanding mechanisms driving arthropod attraction to carrion, and the importance of non-arthropod decomposers (such as microbes) have only recently begun to be investigated (Crippen *et al.* 2015). Evaluating the drivers of arthropod attraction, and the role of numerous ecosystem players in this initial attraction will provide much-needed insight into the factors determining the pre-colonization interval, and the subsequent patterns of arthropod succession that follow.

Patterns of arthropod succession on carrion have been studied on various animals as model organisms and in a number of environments, across different seasons, across geographic regions, and within different ecosystems (e.g., aquatic ecosystems) (Anderson and VanLaerhoven 1996, Anderson 2001, Arnaldos *et al.* 2004, Hobischak and Anderson 2002); but the mechanisms driving the resulting variation remain poorly understood. In addition to understanding the timing and arrival of numerous carrion-associated arthropods, how the carrion is actually utilized by these organisms must also be considered. Some organisms, such as many species in the Calliphoridae (Diptera), depend almost solely on the carrion resource to meet their nutritional requirements for growth and development (Byrd and Castner 2010), while others are facultatively predatory and can prey on calliphorid larvae as well as consuming the carrion tissue (Brundage *et al.* 2014). Furthermore, there are organisms that are commonly noted in close association with carrion, but do not utilize the remains, such as house flies, *Musca domestica* Linnaeus (Diptera: Calliphoridae) (Chin *et al.* 2008), and numerous other incidental species, including spiders (Araneae), wasps (Hymenoptera), moths and butterflies (Lepidoptera), and pill bugs (Armidillidae), which may be associated with the remains as an extension of their own habitats (Byrd and Castner 2010).

Understanding the mechanisms driving arthropod succession on carrion, and the consequences of variations in succession patterns, may lead to a greater understanding of the utilization of vertebrate remains by invertebrate consumers, and allow for more precise and accurate evaluations of time of colonization estimates for forensic investigations. My research aimed at evaluating the volatile organic compounds (VOCs) produced by vertebrate remains under a range of conditions, and assess invertebrate responses to these remains. Two species of fly commonly associated with carrion were utilized for study, each thought to occupy different temporal and feeding niches on carrion. *Cochliomyia macellaria* (Fabricius) (Diptera: Calliphoridae) can arrive at carrion within minutes of environmental exposure, making it a primary carrion colonizer (Anderson 1995, Hall and Doisy 1993), and is heavily reliant on vertebrate remains for its growth and development, making it a specialist in terms of having a narrow dietary breadth (Byrd and Castner 2010). *Hermetia illucens* (Linnaeus) (Diptera: Stratiomyidae) has been most frequently documented at carrion in the later stages of decomposition, making it a secondary colonizer (Dunn 1916, Lord *et al.* 1994). But it is able to utilize manure, carrion and decaying plant matter to meet its developmental requirements, making it a generalist feeder (Sheppard *et al.*

2002). Oviposition on a carcass was used as a measure of attraction and response in this study to assess the attraction and temporal preferences of these species.

5.2 Findings

Volatile organic compound emission analysis from variably aged, colonized and uncolonized carcasses (chapter 2) allowed for an assessment of the classes and origin of VOCs emitted during the decomposition process, both with and without the presence of an actively feeding, primary (chapter 3) and secondary (chapter 4) colonizer, as well as emissions from decaying plant matter. The overlap of compounds emitted from colonized and uncolonized rat carcasses, and the grain diet (such as dimethyl disulfide, decane, nonanal and acetophenone), provide a proposed explanation as to the attractiveness of these substrates to both *C. macellaria* and *H. illucens.* Significant differences between decomposition treatments ($p < 0.05$) indicate that the presence of a primary colonizer significantly changes the composition and abundance of VOCs from vertebrate remains, and may thus have an impact on the attractiveness of the remains to other carrion insects.

The oviposition responses of *C. macellaria* (chapter 3) were significantly greater on carcasses that had been decomposing for 5 d, regardless of colonization status or exposure time. This species has historically been described only as a primary colonizer (Brundage *et al.* 2014, Pechal *et al.* 2014, Tomberlin and Adler 1998, Tomberlin *et al.* 2005), arriving at remains during the early stages of decomposition and co-existing with other primary colonizers. However, the results presented here propose that while *C. macellaria* can be facultatively either primary or secondary colonizers, colonizing vertebrate remains during early or alter stages of decomposition and without consideration of prior colonization. The driving factor in attraction and colonization appears to be carcass age; older carcasses have a greater chance of having been mechanically broken down by the larvae of those species that arrived first (Hobson 1932), and a large portion of muscle tissue has been broken down into simpler compounds by microbial metabolism (Thompson *et al.* 2013). These factors serve as pre-digestion mechanisms, making digestibility by *C. macellaria* larvae easier (Benbow *et al.* 2015). A further benefit to the utilization of an older carcass may also be competition avoidance; in ideal conditions, blow fly larvae do not spend more than a few days on a carcass before migrating to pupate (Byrd and Allen 2001), so colonizing at the later stages after these larvae have vacated the carcass decreases the prospect of interspecific competition. This approach does, however, leave larvae at the risk of having less of the resource available for consumption, due to a large mass of the carcass having already been consumed (Matuszewski *et al.* 2014).

Hermetia illucens (chapter 4) exhibited an oviposition preference to an aged carcass, with colonization status not being a significant driving factor in this choice. However, there was a significantly higher oviposition response when a grain-based diet was introduced, indicting the propensity of this species to possibly preferentially utilize decaying plant matter rather than vertebrate remains. This species has been observed at carrion both during the early stages of decomposition (Tomberlin *et al.* 2005), but has most frequently been observed at older remains in the advanced and dry stages of decomposition (Dunn 1916, Lord *et al.* 1994). However, the results of this study revealed a significantly higher oviposition response at a plant-based diet, indicating a potential niche specialization for the decaying plant matter below a carcass. The interface between the carcass and the soil represents an area high in nutrients, with low competitive pressures, and may be contributing to the temporal variation in carrion association observe in this species, as plant matter decays at variable rates, depending on the composition.

The results of this study provided insight into the VOCs resulting from carrion decomposition over time, and allowed a behavioral assessment of two fly species' response to these volatiles under controlled laboratory conditions. VOC overlap between treatments may allow for an explanation of the vast arthropod community composition observed at vertebrate remains, and may also explain the temporal variation in the two species of carrion-associated flies used in this study.

5.3 Application to forensic entomology

Since its inception as a scientific discipline, much of the research in the field of forensic entomology has centered around the physiology of blow fly development, the effects of temperature and tissue type, and the effect of toxicology on development (Boatright and Tomberlin 2010, Byrd and Allen 2001, Campobasso *et al.* 2004). Only in the last two decades has greater emphasis been placed on understanding the ecology of carrion decomposition (Benbow *et al.* 2015, Parmenter and MacMahon 2009) and incorporating this ecology into models that can assist entomologists in providing a more accurate and precision estimate of the time of colonization in legal investigations (Tomberlin *et al.* 2011a, Tomberlin *et al.* 2011b). Studies such as the one presented in this dissertation shed light into the role of microbes in the decomposition process, and the volatiles production associated with microbial presence. Numerous studies have reported the attractive and repulsive capacities of microbes to blow flies (Barnes *et al.* 2010, Ma *et al.* 2012), and the data presented here that specify the compounds produced under varying conditions of decomposition allow for a more complete picture of microbe succession during decomposition by tracking the changes in compound production and abundance over time (Forbes *et al.* 2014).

Entomologists often use arthropod succession patterns on carrion to make inferences about time of colonization of vertebrate remains, since succession is assumed to occur in predictable patterns (VanLaerhoven and Anderson 1999, Wang *et al.* 2008). However, the findings of the present research show that variability in successional patterns could be due to misconceptions about arthropods that colonize carrion in predictable fashion (e.g., *C. macellaria* colonizes carrion immediately at death, which is inferred). I suggest caution be exercised when assuming that the species present arrived as primary colonizers of the carcass. As such, time of colonization estimates based solely on species presence should be avoided, as these can be misleading and may lead to utility of species that arrived later than anticipated, thus skewing the time of colonization (TOC) estimate. Furthermore, the role of the species in the given ecosystem and its feeding guild should also be carefully considered. Species with a narrow dietary breath (specialists), such as the Calliphoridae, are more likely to be significantly dependent on vertebrate remains, and should thus be given preference for utilization in time of colonization estimates. However, species with a wider dietary breadth (generalists), such as the black soldier fly, may have specific dietary needs that are not met by carrion alone. In addition to biological differences in life history traits from the Calliphoridae, soldier flies may be associated with carrion by attraction, but not necessarily feeding on the remains. Consequently, caution should be exercised when using non-specialist species to infer time of colonization estimates. Nonetheless, the ecological role of generalist species on carrion must be considered as niche specialists; the ability to exploit a previously unexplored niche is, in itself, a reason to potentially redefine the ecological role of this species. On a large scale, black soldier fly larvae may be generalist feeders, but on the finer scale of carrion itself, they are indeed, niche specialists, redefining the carrion ecosystem.

5.4 Potential limitations

While studies such as this one provide extensive insight into the ecological relationships on carrion and the mechanisms driving arthropod succession, all laboratory studies are somewhat limited in their applicability to case work for legal investigations. One such limitation is the assumption that laboratory-reared and wild-type populations behave in the same way. Both populations used in this study have been reared for numerous generations in a laboratory setting, with an infrequent introduction of new genetic material. The conditions of experimentation for these studies were strictly controlled, with known temperature, humidity and lighting regimes. All abiotic factors, such as temperature and moisture content, were kept at near optimal conditions for each species (Boatright and Tomberlin 2010, Meneguz *et al.* 2018), and apart from the parameters being examined, very few external ecological factors were incorporated into these designs. No species interactions were introduced, interspecific competition was removed, and carcasses were left to decompose at lower temperatures than would commonly be observed in this region (College Station, TX) (Zurawski *et al.* 2014). In field conditions, all these factors, in addition to multiple other biotic and abiotic factors, must also be considered. Nonetheless, laboratory studies such as this one provide a framework to begin to understand carrion ecology, such as ecology succession, and lay the foundations for similar studies in the field for more applicable case work data.

The *H. illucens* population used in this study has also been reared for multiple generations on a grain-based diet (standard Gainesville diet). Due to generations of exposure to the same diet, the significant increase in oviposition at the plant-based diet for this species may be a product of an ingrained chemical legacy and predisposition to preference of a plant-based diet, rather than exhibiting a distinct preference over an animal carcass. To clarify whether this is indeed the case, similar studies would need to be performed using a wild population, or one that has been reared on a wider variety of organic waste products.

5.5 Future directions

My research lays a foundation for the assessment and evaluation of carrion age preferences by two fly species, as well as to decipher how these preferences can impact the patterns of arthropod succession observed on carrion. While the results of this study illustrate that carrion age is a significant factor in determining the oviposition preference of these two species, further investigation is needed into the mechanisms driving these preferences. The presence of microbes is critical to decomposition and attraction to carrion, so future work should aim to evaluate the responses of these species in the absence of microbes (i.e., using sterile carcasses) to variably aged carcasses, using only physiological tissue decomposition as a driving factor for attraction. Microbial and VOC isolation can also be used as a tool to evaluate attraction and colonization in the absence of carcasses (Liu *et al.* 2016). Furthermore, electroantennography techniques can be employed to quantify the responses to a range of VOC associated with decomposition (Frederickx *et al.* 2012), particularly those identified in this study as indicator compounds, and those that change over time. Data from such studies could provide an indication of how these organisms respond to the specific chemicals associated with variably aged carcasses, and may allow for a potential prediction of when these species would be most likely to respond to a carcass.

Since the results of this study demonstrate the variation in succession by a species assumed to be solely a primary colonizer, it is necessary to repeat this study using other species also thought to be primary colonizers. Species commonly thought to be primary colonizers, such as *Calliphora vicina* (Meigen), *Lucilia sericata* (Meigen) and *Phormia regina* (Meigen) (all Diptera:

Calliphoridae) (Berg and Benbow 2013, Singh and Bharti 2001, Zurawski *et al.* 2014), may demonstrate variation in succession patterns and carcass age preferences. By clarifying the carcass age preference for a range of species, it may be necessary to re-evaluate the patterns of succession observed on carrion, and assess the accuracy and utility of using succession patterns to indicate TOC in criminal investigations. It is also necessary to carefully assess the ecological role of species utilized in TOC estimates; whether the species present are truly utilizing the carrion resource, or whether they are utilizing a resource resulting from the decomposition of the vertebrate carcass, as this may also influence the TOC estimate.

5.6 References

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APPENDIX

SUPPLEMENTARY MATERIAL

Supplementary Table A2.1: PERMANOVA results testing differences in volatile composition across ten days, using three types of decomposing matter: uncolonized rat carcasses, rat carcasses colonized with *Cochliomyia macellaria* larvae, and a standard Gainesville diet. A Bonferonni correction was applied to results. Significant differences are indicated by an asterisk.

Pair	DF	SS	*F*	R^2	*p*-value
0 vs 1	1	0.583	2.369	0.033	0.042
0 vs 2	1	0.446	1.845	0.026	0.080
0 vs 3	1	1.106	4.223	0.057	0.001^*
0 vs 4	1	1.778	6.768	0.088	0.001^*
0 vs 5	1	2.292	8.293	0.106	0.001^*
0 vs 6	1	2.044	7.372	0.095	0.001^*
0 vs 7	1	2.435	8.643	0.110	0.001^*
0 vs 8	1	2.238	7.888	0.101	0.001^*
0 vs 9	1	2.102	7.432	0.096	0.001^*
0 vs 10	1	2.115	7.431	0.096	0.001^*
1 vs 2	1	0.418	1.638	0.023	0.145
1 vs 3	1	1.189	4.318	0.058	0.004^*
1 vs 4	1	1.695	6.139	0.081	0.001^*
1 vs 5	1	1.851	6.391	0.084	0.001^*
1 vs 6	1	1.663	5.722	0.076	0.001^*
1 vs 7	1	1.926	6.529	0.085	0.001^*
1 vs 8	1	1.832	6.166	0.081	0.001^*
1 vs 9	1	1.710	5.773	0.076	0.001^*
1 vs 10	1	1.735	5.823	0.077	0.001^*
2 vs 3	1	0.584	2.153	0.030	0.043
2 vs 4	1	1.158	4.259	0.057	0.001^*
2 vs 5	1	1.495	5.237	0.070	0.001^*
2 vs 6	1	1.280	4.469	0.060	0.002^*

Supplementary Table A2.1 Continued

Pair	DF	SS	*F*	R^2	*p*-value
2 vs 7	1	1.628	5.600	0.074	0.001^*
2 vs 8	1	1.483	5.065	0.067	0.001^*
2 vs 9	1	1.396	4.781	0.064	0.001^*
2 vs 10	1	1.413	4.810	0.064	0.001^*
3 vs 4	1	0.200	0.686	0.010	0.684
3 vs 5	1	0.518	1.694	0.024	0.077
3 vs 6	1	0.451	1.473	0.021	0.159
3 vs 7	1	0.686	2.207	0.031	0.040
3 vs 9	1	0.706	2.255	0.031	0.048
3 vs 9	1	0.657	2.104	0.029	0.045
3 vs 10	1	0.681	2.170	0.030	0.039
4 vs 5	1	0.238	0.776	0.011	0.590
4 vs 6	1	0.236	0.767	0.011	0.599
4 vs 7	1	0.390	1.250	0.018	0.245
4 vs 8	1	0.566	1.805	0.025	0.083
4 vs 9	1	0.571	1.825	0.025	0.086
4 vs 10	1	0.590	1.876	0.026	0.086
5 vs 6	1	0.070	0.219	0.003	0.998
5 vs 7	1	0.110	0.338	0.005	0.973
5 vs 8	1	0.276	0.844	0.012	0.537
5 vs 9	1	0.282	0.864	0.012	0.490
5 vs 10	1	0.329	1.001	0.014	0.396
6 vs 7	1	0.068	0.207	0.003	1.000
6 vs 8	1	0.158	0.482	0.007	0.839
6 vs 9	1	0.136	0.415	0.006	0.913
6 vs 10	1	0.178	0.542	0.008	0.798
7 vs 8	1	0.129	0.388	0.006	0.941
7 vs 9	1	0.150	0.453	0.006	0.889
7 vs 10	1	0.189	0.567	0.008	0.800

Supplementary Table A2.1 Continued

Pair	DF	SS	*F*	R^2	*p*-value
8 vs 9	1	0.064	0.191	0.003	0.998
8 vs 10	1	0.035	0.105	0.002	1.000
9 vs 10	1	0.026	0.076	0.001	1.000

Supplementary Table A2.2: Biological origin and function of indicator compounds detected from analysis of decomposing uncolonized rat carcasses, rat carcasses colonized with *C. macellaria* larvae, and Gainesville diet.

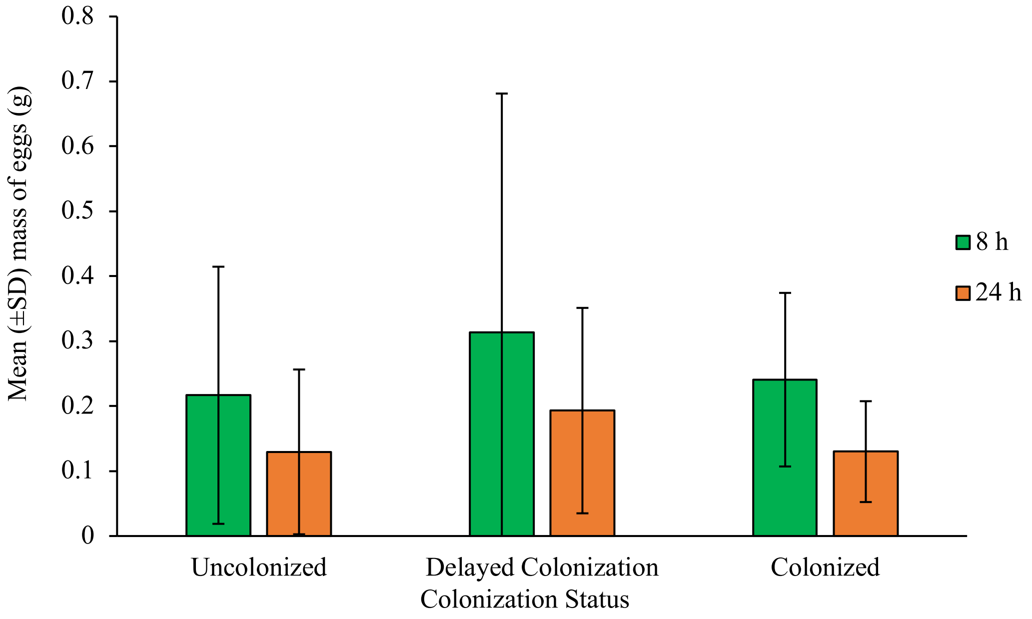
Compound	Microbial origin	Biological function	Reference
Methyl isovalerate	Bacterial & fungal	Antifungal and inhibition of nematocidal activity	(Xu and Luan 2015)
1-Bromo-2-methoxybenzene	Unknown	Unknown	n/a
4-Ethyl phenol	Bacterial	Fungal xenobiotic metabolite	(Ercolini *et al.* 2009)
Indole	Bacterial & fungal	Regulate biofilm formation	(Schulz and Dickschat 2007)
Trimethyl pyrazine	Bacterial & fungal	Fruit fly attractant	(Schulz and Dickschat 2007)
2-Octanamine	Unknown	Unknown	n/a
Phenylethyl alcohol	Bacterial & fungal	Nematocidal activity; wasp attractant	(Davis *et al.* 2012, Xu and Luan 2015)
Naphthalene	Fungal	Unknown	(Buśko *et al.* 2014)
2-Undecanone	Bacterial & fungal	Inhibit fungal growth; nematocidal activity	(Schulz and Dickschat 2007, Xu and Luan 2015)
Methyl pyrazine	Bacterial & fungal	Fruit fly attractant	(Schulz and Dickschat 2007)
Hexadecane	Bacterial	Unknown	n/a

Supplementary Table A2.2 Continued

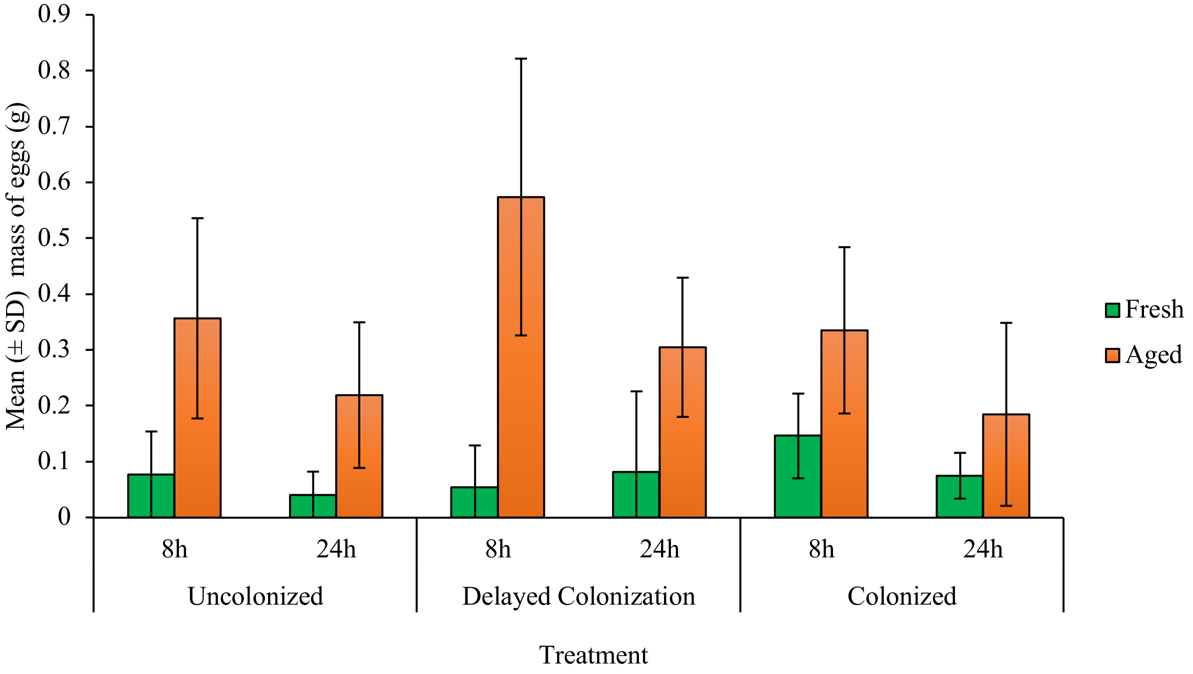
Compound	Microbial origin	Biological function	Reference
Hexanoic acid	Bacterial	Unknown	n/a
Nonadecane	Bacterial	Unknown	n/a
Tetradecane	Bacterial	Unknown	n/a
Dimethyl tetrasulfide	Bacterial	Unknown	(Schulz and Dickschat 2007)
4-Methyl quinazoline	Bacterial	Unknown	(Blom *et al.* 2011)
Butylated hydroxytoluene	Bacterial	Inhibits mycelial growth	(Gong *et al.* 2015)
Diethyl phthalate	Fungal	Unknown	(Buśko *et al.* 2014)
3-Ethyl-2,5-dimethylpyrazine	Bacterial	Protection against soil-borne pathogens	(Sheoran *et al.* 2015)
Aromadendrene	Fungal	Unknown	n/a
1-Octen-3-ol	Bacterial & fungal	Inhibits *A. thaliana* development	(Splivallo *et al.* 2007)
2-Heptanone	Bacterial & fungal	Microbial growth inhibitor; Nematocidal activity	(Xu and Luan 2015)
2-Pentylfuran	Fungal	Unknown	n/a
Dimethyl disulfide	Bacterial & fungal	Nematocidal; kills pathogens; bacteriostatic	(Xu and Luan 2015)
Decane	Bacterial & fungal	ISR in *A. thaliana*	(Ryu *et al.* 2004)
Acetophenone	Bacterial & fungal	Growth inhibition/death of microbes	(Gu *et al.* 2007)
Acetoin	Bacterial & fungal	Attractant for *A. ludens* (Diptera)	(Schulz and Dickschat 2007)
Nonanal	Bacterial & fungal	Inhibits fungal growth	(Schulz and Dickschat 2007)
1-Methyl-1H-pyrrole	Bacterial	Inhibits *A. flavus*	(Gong *et al.* 2015)

Supplementary Table A2.2 Continued

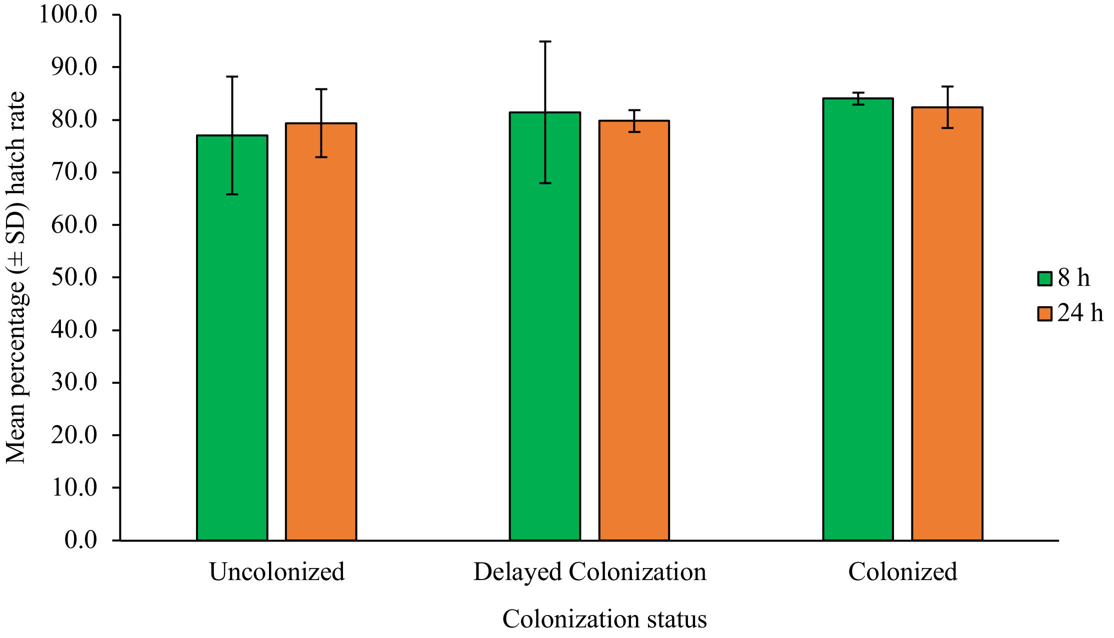
Compound	Microbial origin	Biological function	Reference
1-Hexanol	Bacterial & fungal	Unknown	n/a
2-Methyl-ethyl-ester-butanoic acid	Bacterial & fungal	Unknown	n/a
2,4,7,9-Tetramethyl-5-decyn-4,7-diol	Bacterial	Unknown	n/a



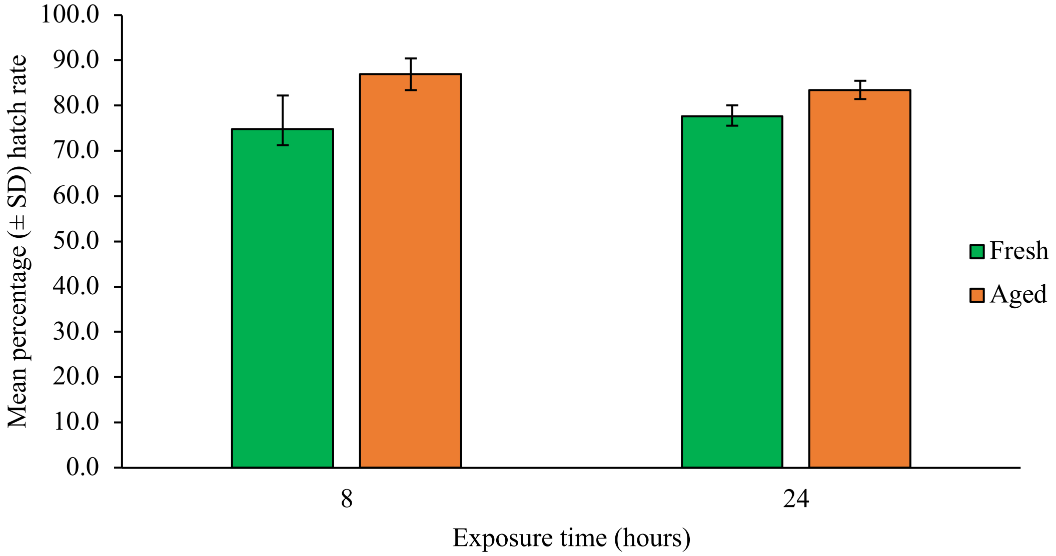
Supplementary Figure A3.1: Effect of exposure time and colonization status on the mean (± SD) mass of eggs (grams) oviposited by *C. macellaria* females held at 27°C and 65% RH.



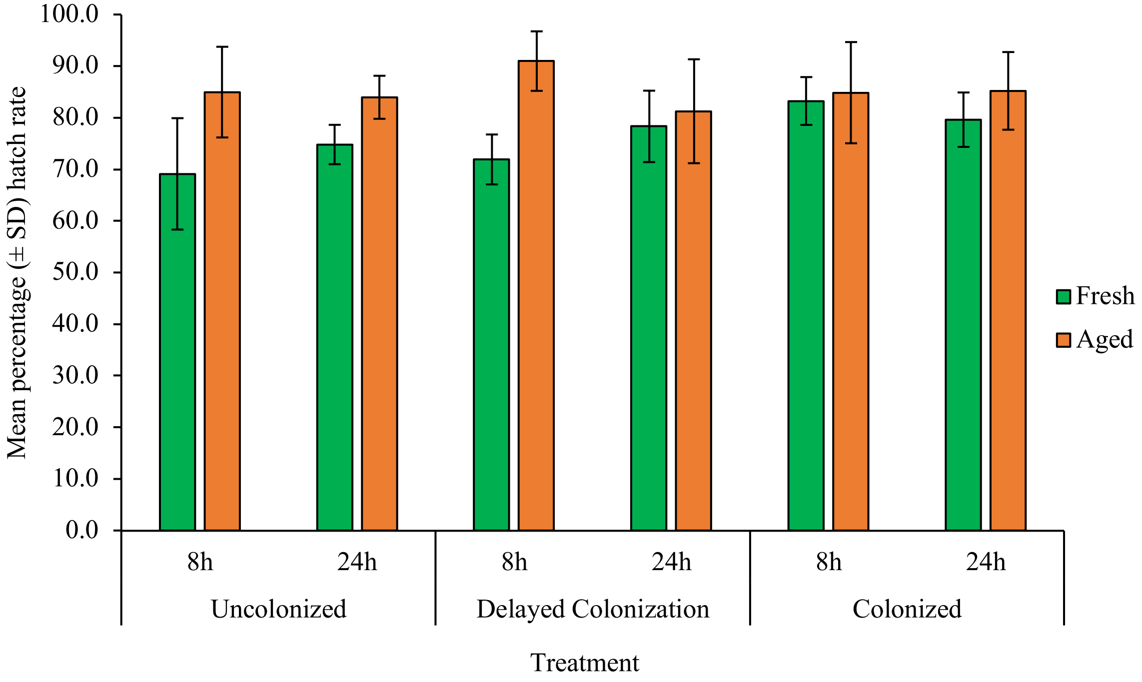
Supplementary Figure A3.2: Effect of colonization status, exposure time and carcass age on the mean (± SD) mass of eggs (grams) oviposited by *C. macellaria* females held at 27°C and 65% RH.



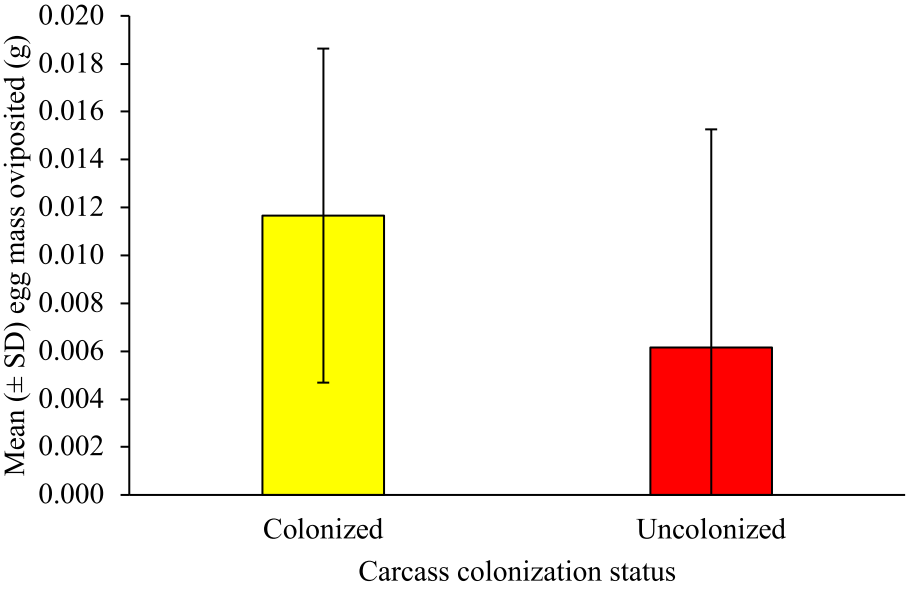
Supplementary Figure A3.3: Effect of colonization status and exposure time on the mean percentage (± SD) of eggs hatching from eggs oviposited by *C. macellaria* adults held at 27°C and 65% RH.



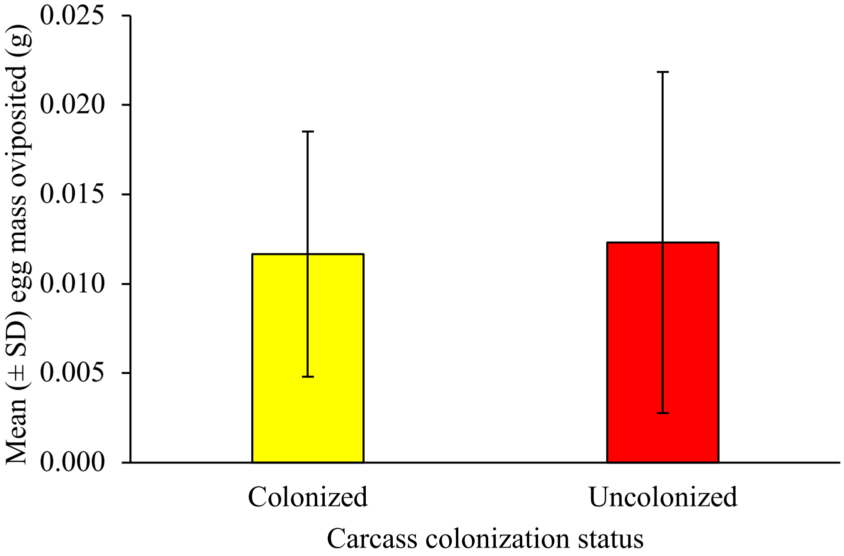
Supplementary Figure A3.4: Effect of exposure time and carcass age on the mean percentage (± SD) of eggs hatching from eggs oviposited by *C. macellaria* adults held at 27°C and 65% RH.



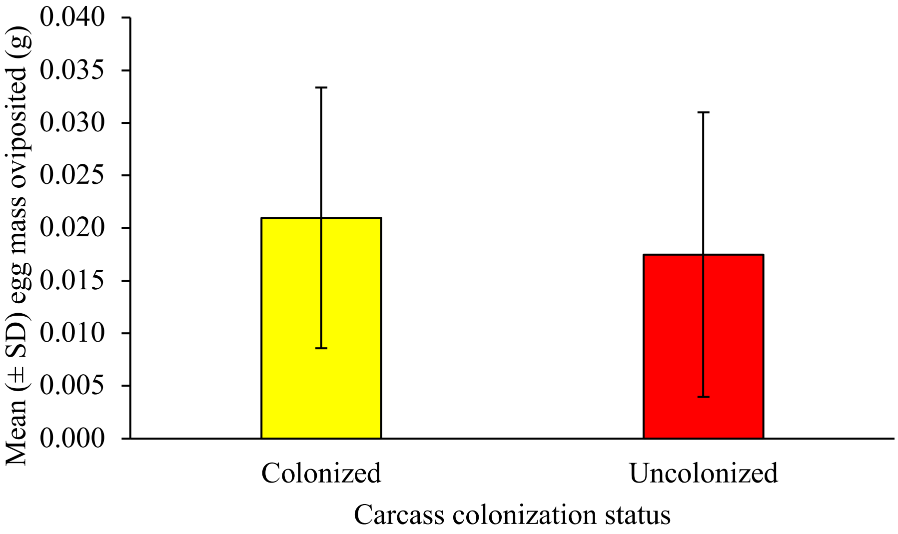
Supplementary Figure A3.5: Effect of colonization status and exposure on the mean percentage (± SD) of eggs hatching from eggs oviposited by *C. macellaria* adults held at 27°C and 65% RH.



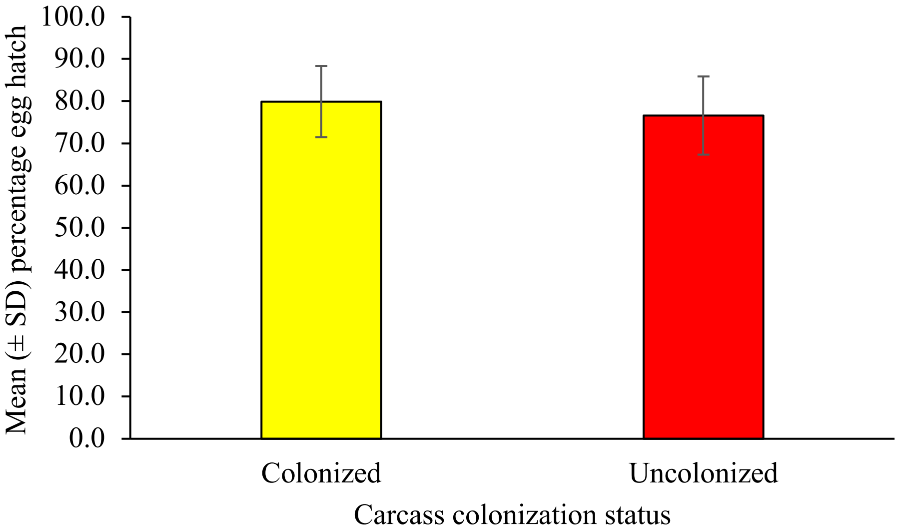
Supplementary Figure A4.1: Mean (± SD) mass of eggs oviposited by *H. illucens* adults at rat carcasses colonized with a primary colonizer, *C. macellaria*, and uncolonized rat carcasses, after an exposure time of 8 h, taken as a function of all treatments, including those where no oviposition activity was recorded. Figure represents untransformed data.



Supplementary Figure A4.2: Mean (± SD) mass of eggs oviposited by *H. illucens* adults at rat carcasses colonized with a primary colonizer, *C. macellaria*, and uncolonized rat carcasses, after an exposure time of 8 h, taken as a function of treatments only where oviposition activity was recorded. Figure represents untransformed data.



Supplementary Figure A4.3: Mean (± SD) mass of eggs oviposited by *H. illucens* adults at rat carcasses colonized with a primary colonizer, *C. macellaria*, and uncolonized rat carcasses, after an exposure time of 8 h, taken as a function of treatments only where oviposition activity was recorded, and treatments that included both rat carcasses and Gainesville diet. Figure represents untransformed data.



Supplementary Figure A4.4: Mean (± SD) percentage hatch from clutches of eggs oviposited by *H. illucens* adults at rat carcasses colonized with a primary colonizer, *C. macellaria*, and uncolonized rat carcasses, after an exposure time of 8 h, taken as a function of treatments only where oviposition activity was recorded, and treatments that included both rat carcasses and Gainesville diet. Figure represents untransformed data.

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