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

A Dissertation

by

ADRIENNE LEANE BRUNDAGE

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

DOCTOR OF PHILOSOPHY

May 2012

Major Subject: Entomology

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Approved by:

Chair of Committee,	Jeffery K. Tomberlin
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ABSTRACT

Fitness Effects of Colonization Time of *Chrysomya rufifacies* and *Cochliomyia macellaria,* and their Response to Intra- and Inter-specific Eggs and Egg-Associated Microbes. (May 2012)

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Chrysomya rufifacies and *Cochliomyia macellaria* are two medically and forensically important necrophagous flies that dominate ephemeral resources in the southern US. Since its introduction in 1981, *Ch. rufifacies* has become established throughout the New World due to its larvae being facultative predators. Through this research I examined the interaction between the native, primary colonizer *C. macellaria* and the invasive, secondary colonizer *Ch. rufifacies* and elucidated the olfactory mechanisms used to locate, colonize, and exploit ephemeral resources. This work used competition experiments, olfactometer experiments, and high-throughput sequencing to investigate the effects of priority colonization of ephemeral resources on both species, the olfactory mechanisms employed by gravid females to locate a resource, and the effects of egg-associated volatiles on those females.

Results from competition experiments indicated that priority sequence significantly affected the fitness of both *C. macellaria* and *Ch. rufifacies*. Regulation of

colonization time is not chiefly governed by resource age, as previously thought, but is affected by colonization of the resource by conspecific and heterospecific individuals. Colonizing adults may use cues from early colonizers to assess resource quality. These cues may be derived from the physiology of the eggs, the 31-39 species of bacteria I determined are present on the egg chorion, or some combination of both. Design of these experiments facilitated the development of techniques to surface-sterilize Calliphoridae eggs, analyze behavior of adults in a dual choice olfactometer, and associate adult response to conspecific and heterospecific eggs with environmental cues that ultimately affect larval fitness.

DEDICATION

I would like to dedicate this dissertation to the most supportive and amazing husband in the world. Dean, I could not have done this without you.

ACKNOWLEDGEMENTS

First off, I'd like to thank my advisor, Dr. Jeff Tomberlin, for initially recruiting me to the fabulous F.L.I.E.S. facility, and then giving me not only the freedom to work through my research ideas, but the support and encourgement I needed to get me through. Thank you, Jeff, sincerely. I can't wait to see what the future holds for us!

I would also like to thank members of my committee, Dr. Micky Eubanks, Dr. Craig Coates, and Dr. Michael Manson. You each gave me hours of your precious time to make my research as great as possible.

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To my friends and family: thank you for giving me your support during this process, your understanding every time I had to cancel plans or wanted to sleep, and your unconditional love as I became a vibrating ball of nerves. Your presence in my life made this process bearable, even when it seemed like a never-ending process. I especially want to thank Jill and Rayna—our weekly lunches were the bright spot in a long period of stressful days, and just knowing I'd have a few minutes to kick back each week got me through. I love you guys.

To my mom and dad: thank you guys, again and again, for first encouraging me to take this position, and then supporting us when we needed it. Those long trips through

Texas for a short Christmas visit meant the world (and the longer trips to the grocery store to make sure we had food didn't hurt). I hope I have made you proud.

Finally, to my husband Dean. It's been a very rocky road over the past four years. You uprooted your life for me, followed me to a place we never dreamed of living, and have stood by me through thick and thin. I think I told you once that I couldn't do this without you, and that was the truth. Your support, your love, your over all goodness has made this possible. I will be forever grateful. I love you more than you could ever know, despite how many times I insist on telling you every day. Thank you, from the bottom of my heart.

This has been a crazy rollercoaster. I want to tell you the story of how I first got this position. I was minding my own business at a conference when I got offered a doctoral position. I talked it over with Dean and my family, and we decided we were going to go to Texas! Of course, when I told my mom, she got scared and said "you're moving with your auntie and uncle in Bel-Air." I whistled for a cab and when it came near the liscense plate said fresh and it had dice in the mirror. If anything I could say this cab was rare, but I thought, "Nah, forget it. Yo homes to Bel-Air!" I pulled up to the house about 7 or 8 and I yelled to the cabby "Yo homes smell ya later!" Looked at my kingdom I was finally there, to sit on my throne as the prince of Bel-Air (Smith 1990).

I also like to give a special thanks to Mountain Dew and green tea. Your caffeinated goodness got me through this.

NOMENCLATURE

ADBAC	Benzalkonium Chloride
CFU	Colony Forming Units
D	Day
EtOH	Ethanol
H	Hour
H_2CO	Formaldehyde
L:D	Light: Dark Cycle
MDT	Maggot Debridement Therapy
m-PMI	Minimum Post Mortem Interval
NaOCl	Sodium Hypochlorite (Clorox®)
OTU	Operational Taxonomic Unit
PIA	Period of Insect Activity
PMI	Post Mortem Interval
RH	Relative Humidity
RO	Reverse Osmosis
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
VOC	Volatile Organic Compound

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Nutrients cycle through the ecosystem via two major pathways: grazing and the detritus food chain (Cornaby 1974). Herbivores only consume a small portion of the above ground primary production. Decomposers, detritivores, or necrophages consume the balance, including the carcasses of members of higher tropic levels (Whitford 1986). Nutrients enter the decomposer subsystem via leaf, seed and fruit drop, animal defecation, and vertebrate and invertebrate death (carrion). These ephemeral patches are discrete, undivided habitat resources with short durational stability (Beaver 1984) Necrophagous bacteria, fungi and animals release the nutrients from the dead material back into the ecosystem (Seastedt 1984), and these organisms are therefore an important part of energy flow throughout the bionetwork. Carrion has been studied extensively as an ephemeral habitat (Beaver 1977, Byrd 1998), as it supports a diverse community of necrophagous insects due to its status as a high quality nutrient source (Parmenter and Lamarra 1991).

Arthropods regulate the carrion decomposition pathway (Parmenter and Lamarra 1991) by advancing decomposition and recycling bound nutrients back into the system (Barot et al. 2007). In the absence of arthropods, carrion decomposition rates are significantly slower than when arthropods are present (Parmenter and MacMahon 2009).

This dissertation follows the style of Journal of Medical Entomology.

For example, Parameter and Macmahon (2009) observed a 90% decrease in decomposition rates of carrion when arthropods were eliminated from the process than when arthropods were granted access. The arthropods primarily responsible for decomposition are members of the orders Diptera and Coleoptera. Of the approximately 1,000 dipteran species in North America over 100 are associated with carrion (Catts and Haskell 2008). The sheer number of species coupled with their voracious consumption of decomposing animal tissue makes the Diptera the most important insect order in terms of carrion reduction (Cornaby 1974, Goff 1993, Catts and Haskell 2008).

Flies inhabit two separate tropic levels: that of primary consumer as adult, and that of decomposer as a larva. Gravid adults are attracted to decomposing organisms, and lays eggs on or near the resource. Rapid decomposition of a high quality resource results in intense intraspecific and interspecific competition among dipteran larvae (Lang et al. 2006). Such competition affects the participants. For example, interspecific competition between *Chrysomya albiceps* (Wiedemann) (Diptera: Calliphoridae) and *Lucilia sericata* (Meigen) (Diptera: Calliphoridae) resulted in decreased survivorship, adult size and fecundity of the inferior competitor (Kheirallah et al. 2007). This pattern tends to hold true across most carrion-breeding species: as larval numbers increase on a resource, adult size decreases (Lang et al. 2006). The implication of such results is that competitors for ephemeral resources should evolve mechanisms to avoid the detrimental effects of competition.

Blow flies (Diptera: Calliphoridae) are generally the first arthropods to colonize carrion (Byrd 1998). While approximately 1,000 species in 150 genera are known

(Whitworth 2006), 10 carrion-inhabiting blow fly species are known in Texas (McAlpine 1981, Tenorio et al. 2003). *Calliphora vicina* (Robineau-Desvoidy) (Diptera: Calliphoridae) and *Phormia regina* (Meigen) (Diptera: Calliphoridae) colonize fresh remains during cool months in Texas (November through February) (Tenorio et al. 2003, Bucheli et al. 2009), while *Chrysomya rufifacies* (Macquart) (Diptera: Calliphoridae) and *Cochliomyia macellaria* (Fabricius) (Diptera: Calliphoridae) are the dominant colonizers of carrion during warm months (Tenorio et al. 2003).

Cochliomyia macellaria was first described in 1775 and is known as the secondary screwworm due to its ability to cause secondary myiasis (Baumgartner 1985). The metallic, green-blue adults are identifiable by three longitudinal stripes on the dorsal thorax and yellow genal dilation (Whitworth 2006). It is native to the New World temperate and tropic regions from Canada through Argentina (Whitworth 2006). It prefers warm environments (Byrd and Butler 1996) and under ideal conditions will disperse from southern to northern areas. (Whitworth 2006).

Developmental time and activity of this species is temperature dependent. Eggs have a low temperature threshold of 10-12.5^{0}C while larvae pass through three instars and take 588 h at 15^{0}C to 170 h at 32^{0}C to complete development (Byrd and Butler 1996). Adults mate between 3 and 18 d post eclosion, and females can oviposit up to eight egg masses on carrion over their lifetime (Baumgartner 1985). It is considered hemi- to eusyanthropic depending on human population density (Baumgartner 1993).

Cochliomyia macellaria is considered a primary colonizer of carrion (Campobasso and Introna 2001). Campobasso (2001) determined *C. macellaria* to be the first to

colonize human remains in Tennessee during summer month. Similarly, Gruner et al. (2007) collected arriving adults minutes after pig carcass placement. Initial decomposition studies on human remains placed at the Forensic Anthropology Research Facility at Texas State University documented *C. macellaria* to be primary dipteran colonizer in the spring (Pechal, unpublished data.).

Cochliomyia macellaria populations have diminished in Brazil and Argentina (Reis et al. 1999), due to invasive *Chrysomya* species. *Cochliomyia macellaria* is an inferior competitor in the presence of *Chrysomya putoria* (Wiedemann) (Diptera: Calliphoridae) and *Ch. rufifacies* although coexistence between the species does occur (Reis et al. 1999). Rather than engage in scramble competition normally seen in blow flies (Rosa et al. 2006), *Ch. rufifacies* is facultatively predacious and uses *C. macellaria* larvae as an additional food source (Wells and Greenberg 1992b, Rosa et al. 2006). *Cochliomyia macellaria* attempts to avoid predation by either leaving the resource prematurely or burrowing into the resource (Wells and Greenberg 1994).

Chrysomya rufifacies was described in 1843 and is known as the hairy maggot blow fly due to characteristic fleshy protrusions on the larvae (Baumgartner 1993). Adults are metallic green-purple with pale gena (Whitworth 2006). This species is indigenous to the Old World (Roy and Siddons 1939), but has recently been introduced into several new world countries.

Chrysomya rufifacies second and third instar larvae are facultative predators and cannibals (Goodbrod 1990). In laboratory trials, *Ch. rufifacies* can switch from necrophagy to cannibalism and predation when confronted with limited resources

(Goodbrod 1990). This ability allows *Ch. rufifacies* to continue development despite food depletion (Goodbrod 1990). Wells and Greenberg (1992a) confirmed the predatory ability of *Ch. rufifacies* in a pair-wise predation study with *C. macellaria.* Third instar *Ch. rufifacies* preyed on third and second instar *C. macellaria* larvae in 85% and 35% of the cases respectively (Wells and Greenberg 1992c). Shiao (2008) recorded similar results with third instar *Ch. rufifacies* feeding on *Ch. megacephala*. Watson (2005) observed this predatory behavior by larvae on carrion in Louisiana. *Chrysomya rufifacies* oviposited on the heads of carcasses despite previous oviposition of *C. macellaria* in the same region. Resulting *C. macellaria* larvae dispersed to peripheral areas of the carcass or left it all together (Watson 2005).

Chrysomya rufifacies exhibit monogenic reproduction. This phenomenon is rare for Diptera (Roy and Siddons 1939, Subramanian and Mohan 1980) and is determined by maternal genotype (Kirchhoff and Schroeren 1986). Adults mate between 2 and 10 ds after eclosion (Schmidt and Kunz 1985, Baumgartner 1993). Development of mature ovaries is dependent on the ingestion of a protein meal. Females lay between 210 and 368 eggs per clutch (Schmidt and Kunz 1985). Developmental time of this species is dependent on temperature and perhaps latitude (Schmidt 1989, Byrd and Butler 1997). Eggs have a lower temperature threshold of 9^0C (Baumgartner 1993). Resulting larvae pass through three instars and take 600 to 200 h at 5 and 32^0C respectively to develop from egg to adult (Byrd and Butler 1997). Adult flight activity has a minimum threshold of 13^0C (Baumgartner 1993), and live approximately 23-30 d (Schmidt and Kunz 1985). This species was thought to be active during warm weather (Baumgartner 1993).

However, researches recently collected adults during November in South Carolina with the temperature ranging from 8.2^0C to 12.8^0C (Cammack and Nelder 2010).

The hairy maggot can cause myiasis, the infestation of live animals with Diptera larvae (Byrd and Castner 2010). The first incidence of *Ch. rufifacies* myiasis reported in the United States was on a dog in 1983, and several cases on animals and humans in Brazil have been documented as well (Baumgartner 1985). *Chrysomya rufifacies* is able to cause sheep strike and is an important parasite of new born calves in wet areas of Hawaii (Wells and Greenberg 1992c). The bulk of *Ch. rufifacies* interactions with vertebrate animals is on carrion, however, and the first maggots were discovered in the U.S. in 1981 on a dead cheeta (Baumgartner 1985).

In its native range, *Ch. rufifacies* is considered a secondary carrion fly (Fuller 1934b, Chin 2007). Chin (2007) reported that in Malaysia, *Ch. megacephala* is the first fly to appear in a larval mass, but *Ch. rufifacies* will assume the dominant role within 48 h. In areas where it is an introduced species, *Ch. rufifacies* reportedly behaves as a primary carrion fly (Chin 2007, Gruner et al. 2007).

Cochliomyia macellaria and *Chrysomya rufifacies* not only compete for resource, but interact as predator and prey (Lima and Dill 1990). As previously discussed, *Ch. rufifacies* larvae will depredate *C. macellaria* larvae or drive them from carrion (Tenorio et al. 2003, Cammack and Nelder 2010). Baumgartner (1985) found that *C. macellaria* dominance diminished from over 89% of overall blow fly numbers in Peru to 0.2-4% of blow flies following the introduction of *Chrysomya* sp. This observed dominance has prompted entomologists to predict that *Ch. rufifacies* will exclude *C. macellaria* from its

current, native habitats in North America (Wells and Greenberg 1992c, Baumgartner 1993, Rosati 2007).

The mechanism by which *C. macellaria* and *Ch. rufifacies* locate potential food resource is largely unstudied. Certain fly species preferentially oviposit in the presence of conspecific and heterospecific egg masses. Browne et al. (1968) observed 80% more oviposition by *Lucilia sericata* (Meigen) (Diptera: Calliphoridae) in the presence of conspecific eggs (Browne 1958) ; while Rosati and Van Laerhoven (2010) determined that *Phormia regina* (Meigen) (Diptera: Calliphoridae) preferentially oviposited in the presence of *L. sericata* eggs. It is possible that *C. macellaria* and *Ch. rufifacies* exhibit the same oviposition behavior.

Pheromone deposition (Jiang et al. 2002), intraspecific aggregation (Woodcock et al. 2002), and phenological shifts (Fenton 1999) are some strategies allowing carrion flies to avoid competition. Competition inherent to the predator-prey interaction between *Ch. rufifacies* and *C. macellaria* on carrion (Wells and Greenberg 1992c) suggests *C. macellaria* would be selected for traits allowing them to avoid predation by *Ch. rufifacies*.. *Cochliomyia macellaria* might colonize carrion earlier, depart prior to, or avoid carrion already inhabited by *Ch. rufifacies*. Lam et al. (2007) determined muscid flies (Diptera: Muscidae), utilize cues released by bacteria as an oviposition attractant or deterrent, depending on bacterial density (Lam et al. 2007). Since *C. macellaria* and *Ch. rufifacies* are known necrophages, they have evolved in the presence of extensive bacterial communities. It is possible that *C. macellaria* use resource-associated bacteria to evaluate potential oviposition sites and predation risk. Conversely, *Ch. rufifacies* may

use similar cues to evaluate oviposition substrates and locate prey.

Bacteria are closely associated with eukaryotic organisms (Dale and Moran 2006). Bacteria exhibit different ecological roles with arthropods ranging from parasite to mutualist and commensalist. However, microbial effects on insects at both the species and community levels have been largely understudied (Weinert et al. 2007), although Janzen (1977) suggested microbial communities compete with macrobiotic organisms for transient resources (Janzen 1977). Recently, research has examined competition between the microbial and animal kingdoms (Deron et al. 2006), and direct mutualism in the form of endosymbionts (Nardi et al. 2002). Outside of parasitic context (Eddy 1975), there is little work on the inter-kingdom communication between prokaryotic and eukaryotic organisms as it relates to resource and habitat location, suitability, and colonization.

Bacteria are intimately associated with the decomposition of human remains (Perper 1993). Putrefaction of the remains by associated bacteria begins at death of the individual, resulting in the production of ammonia compounds and hydrocarbons (Fiedler 2003). Baumberger (1919) postulated that bacteria drive insect succession, and colonizing insects feed on bacteria as a primary nutritional source (Baumberger 1919). Fuller (1934a) dismissed the idea, stating that decomposition is greatly retarded by insect absence; therefore, insects are the primary driving force behind decomposition (Fuller 1934b). Both researchers acknowledged that bacteria on carrion break down tissues, allowing for nutrient ingestion by colonizing Diptera (Baumberger 1919, Fuller 1934b).

Much more work has been done regarding the bacterial effect on attraction of blow

flies to hosts (Fuller 1934b, Browne 1965, Eddy 1975, Emmens 1982). Emmens (1982) determined *Pseudomonas aeruginosa* degrades wool and produces sulphurous compounds, which attract female *Lucilia cuprina* (Wiedemann) (Diptera: Calliphoridae). Browne (1965) noted *L. cuprina* increased oviposition in response to indole and ammonium carbonate, which are products of bacterial metabolism. Bovine blood inoculated with bacteria is significantly more attractive to the primary screwworm *Cochliomyia homnivorax* (Coquerell) (Diptera: Calliphoridae) than uninoculated blood (Eddy 1975, Chaudhury 2010).

Olfactory cues associated with bacteria induce oviposition in *L. sericata* (Ashworth and Wall 1994). Increased searching and flight times have been noted when females are presented with odors from decomposing meats (Wall 1994). *Lucilia sericata* oviposition is stimulated by the by-products of bacterial breakdown of keratin in wool (Heath and Appleton 2000). Oviposition behavior is activated by ammonium compounds and carbon dioxide, all of which originate with bacterial decomposition of the wool (Ashworth and Wall 1994). While several bacterial species may produce the same odors, it is apparent that the overall species composition of the microbial community on decomposing media greatly affects the attractiveness of the volatiles (Eismann 1988).

Bacteria also served as a source of nutrition for insects. House fly, *Musca domestica* (Linnaeus) (Diptera: Muscidae) larvae reared on media containing bacteria have greater survival and longevity than those on sterile resources (Schmidtmann and Martin 1992). These bacteria also provide growth factors such as sterols and vitamins to the larvae, and house fly larvae reared on sterile blood agar lacking B vitamins have

stunted growth (Brookes and Fraenkel 1958). Bacteria have also been demonstrated to be essential for the growth of *Musca autumnalis* (De Geer) (Diptera: Muscidae) and *Haematobia irritans* (Linnaeus) (Diptera: Muscidae) (Schmidtmann and Martin 1992) which are also colonizers of decomposing material.

Bacteria are considered a major catalyst of insect succession on carrion (Baumberger 1919). Many calliphorid and muscid species use bacteria and their products as food (Brookes and Fraenkel 1958, Ahmad et al. 2006). Calliphoridae cue in on bacterial products to locate feeding and oviposition sites (Cragg 1955). *Cochliomyia macellaria* and *Ch. rufifacies* might therefore use bacteria to facilitate location, colonization, and digestion of a resource.

Elucidating bacteria-blow fly interactions has important implications for decomposition ecology and forensic entomology. Blow flies can arrive and colonize carrion within hours of death. This colonization starts a "biological clock" and age-determination of the developing fly larvae can be used as the basis for estimating how long that carrion has been exposed to insect colonization. (Amendt et al. 2007, Byrd and Castner 2010). Determining the amount of time the larvae have been on the remains can be used to estimate the period of insect activity (PIA) (Tomberlin et al. 2011) which may, in turn, be correlated with time of death (Catts 1992, Byrd and Castner 2010), expressed as the post mortem interval (PMI) provided no delays in colonization (Catts and Haskell 2008, Byrd and Castner 2010, Villet et al. 2010). PIA and inferred PMI are critical pieces of information for death investigators (Villet et al. 2010), and arthropods have been used globally for such determinations (Amendt et al.

2010). Over the past four decades, forensic entomological research has focused on macrobiotic and abiotic influences on the calliphorid life cycle. Oviposition and larval development on scavenged (Campobasso 2001), buried (VanLaerhoven 2008), wrapped (Catts and Goff 1992), and burned (Pai et al. 2007) remains have been extensively studied., Few, however, have examined the role carrion-associated bacteria play in the colonization and consumption of decomposing resources by blow flies. Knowing this information could result in a more refined understanding of niche partitioning and succession patterns of competing blow flies on PIA estimates. My research investigates the exploitation of habitats by two competitive and forensically important blow fly species in central Texas. Specifically, my work investigates determined; 1) the importance of colonization sequence in regards to *C. macellaria* and *Ch. rufifacies* on ephemeral resources; 2) the effect decomposition state of a resource on the attractancy and repellency of *C. macellaria* and *Ch. rufifacies* adults based on sex and ovarian status; 3) the inter- and intra-specific attraction or repellant properties of *C. macellaria* and *Ch. rufifacies* eggs of varying ages; and 4) if egg physiology or associated microbes are responsible for the observed inter- and intra-specific attraction.

CHAPTER II

EFFECTS OF TEMPORAL PRIORITY ON THE LIFE-HISTORY TRAITS OF TWO COMPETING BLOW FLY (DIPTERA: CALLIPHORIDAE) SPECIES ON CARRION

Introduction

Patchy distribution of exploitable resources is widespread throughout nature, and interspecific competition for such patches is often intense. A temporal disparity between ecologically similar species may confer a competitive advantage to primary colonizers under exploitative competition circumstances. An early colonizer is relatively unaffected by the presence of later species, but resource depletion may be a detriment to later-arriving conspecific or interspecific individuals (Bryant 1971, Beaver 1984, Shorrocks and Bingley 1994, Hodge 1996). Competitively inferior species exploiting resources before patch invasion by more competitively dominant species may survive and consequently persist in an environment (Schoener 1974, Hodge 1996). For example, offspring of *Drosophila sp.* (Diptera: Drosophilidae) arriving significantly earlier than ecologically similar species, had a 35% increase in survivorship, 22% decrease in developmental time, and 17% increase in adult size when compared with those resulting from adults arriving later (Hodge 1996). This "priority effect" (Alford and Wilbur 1985) has been documented in other systems such as fish (Geange and Stier 2009), crustaceans (Irving et al. 2007), and amphibians (Eitam et al. 2005). In each case, early-arriving species gained a fitness advantage by exploiting a patch before a competitor arrived. However, it has not been examined in detail for blow flies (Diptera: Calliphoridae).

Priority effect may be responsible for the coexistence of competing Calliphoridae on a carrion resource (Denno 1975, O'Flynn 1983, Schoenly 1992). Primary colonizers of carrion tend to exhibit efficient location and colonization of carrion, along with rapid feeding and growth while on the resource (Beaver 1984). These traits lead to carrion colonizers arriving at a patch early in the decomposition process and devouring the maximum resource possible before competitors arrive (Schoenly 1992). Secondary colonizers must contend with reduced nutrient value and high competitor diversity (Beaver 1984). Natural selection would seemingly therefore favor the earliest and most efficient colonizers of a carcass (Kneidel 1984a, Kneidel 1984b). Secondary colonizers of carrion require an advantage over those already in residence (Lane 1975). Two common hypotheses about such an advantage are the insect must either be an inferior competitor when in direct competition with other species on a resource (Atkinson and Shorrocks 1981) or require the resource to be modified by early colonizers (Lang et al. 2006).

Central Texas has ten commonly occurring species of Calliphoridae (Diptera) that feed on decomposing animal tissue (Tenorio et al. 2003). Of these, *Cochliomyia macellaria* (Fabricius) and *Chrysomya rufifacies* (Macquart) are the most abundant species during the warm months of the year (Goddard 1988, Tenorio et al. 2003, Bucheli et al. 2009). *Cochliomyia macellaria* is native to the New World (Baumgartner 1993) with a distribution from southern Canada, throughout the United States, Mexico, Central America, and southwards to central Argentina. *Cochliomyia macellaria* is a primary colonizer of vertebrate carrion arriving early in the decomposition process (Tomberlin

and Adler 1998). Large collective egg masses may result in >1000 larvae per cm^2 maggot masses, which can quickly devour a cadaver (Laake et al. 1936, Reis et al. 1999, Slone and Gruner 2007, Oliveira and Vasconcelos 2010).

Chrysomya rufifacies is native to Southeast Asia. It was introduced to Central America in 1977 (Baumgartner 1993), arrived in the United States by 1980 (Gagne 1981), and has become well established across North America including southern Canada (Rosati 2007). *Chrysomya rufifacies* acts as a secondary colonizer (Bohart 1951, Norris 1959) arriving at a carcass one to two ds after death (Baumgartner 1993). Larvae are facultatively predaceous in the second and third instars (Fuller 1934b) on *C. macellaria* larvae (Baumgartner 1993). Researchers hypothesize that *Ch. rufifacies* exhibits predatory behaviors when food is scarce due to competition with other necrophagous blow flies (Norris 1965, Goodbrod 1990). This tendency has led researchers to hypothesize that *Ch. rufifacies* will dominate carrion patches and eradicate *C. macellaria* from North America (Wells and Greenberg 1992c, Baumgartner 1993).

Secondary colonization could be a risky behavior for a carrion fly; it increases the likelihood that the resource will become unusable before exploitation by resulting offspring (Beaver 1984) and increases the risk of interspecific competition (So and Dudgeon 1990). Despite these possible risks, *Chrysomya rufifacies* continues to exhibit secondary colonization behavior, which implies an evolutionary benefit.

Since *C. macellaria* and *Ch. rufifacies* exploit carrion as their primary resource, they are considered direct competitors (Kneidel 1984a, Baumgartner 1993, De Jong 1997). Studies documented competition among carrion flies in the field (Denno 1975,

Denno et al. 1995, Archer and Elgar 2003) and in experimental caged populations illustrating some species regularly out compete others to the point of extinction (Hanski and Kuusela 1977). However, these results do not completely apply to *C. macellaria* and *Ch. rufifacies,* since both species continue to persist. The objective of this study was to determine if *Ch. rufifacies* remains as a secondary colonizer due to competition avoidance or through required resource modification, and the effect of arrival times on survivorship, longevity, and fecundity of *Ch. rufifacies* and *C. macellaria.*

Materials and Methods

Adult Fly Colony. Laboratory colonies of *C. macellaria* and *Ch. rufifacies* larvae used in this study were initiated from flies collected in Brazos County, Texas, USA during spring and summer of 2009 and 2010. Larvae were reared on fresh bovine liver provided *ad libitum* in 3 L plastic containers in walk-in growth chamber at 27°C ± 1°C, 60% RH, and a 12:12 (L:D) photoperiod. Dispersing third instar larvae were transferred to 3 L containers with autoclaved sand (Town & Country Landscape Supply Co., Chicago, IL, USA) for pupation. Resulting adults were maintained in 300 cm^3 cages (Bioquip Products, Rancho Dominguez, CA, USA) held in the growth chamber previously described. Granulated sugar (Imperial Sugar Co., Sugar Land, TX, USA), buttermilk powder (Saco foods Inc., Middleton, WI, USA), and water were provided *ad libitum*, and 20 g bovine liver was placed in the cage between 4 and 15 d post emergence for 4 h to induce oviposition as needed.

Experimental Design. Collected eggs were homogenized prior to placement in treatments and controls of this experiment. For mixed species treatments, 100 eggs of the pioneer species were introduced to 100 g of fresh bovine liver in a 20.5-cm x 34.5-cm x 20.5 cm plastic tub with 1.5 L of sand. Eggs were transferred using a camel hair paintbrush. The competing species was introduced 0, 1, 2, 3, or 4 d after introduction of the pioneering species (Fig. 2.1). To ensure that results were not due to resource age, a pure culture of 200 eggs of each species was placed on liver aged 0, 1, 2, 3, or 4 d in the growth chamber under conditions previously described and retained in the same environment (Fig. 2.2). All treatments and controls were established on the same d. All replicates were placed in the rearing room under the conditions previously described. Nine treatments and ten controls were established for this study. Each was replicated a minimum of three times over three generations.

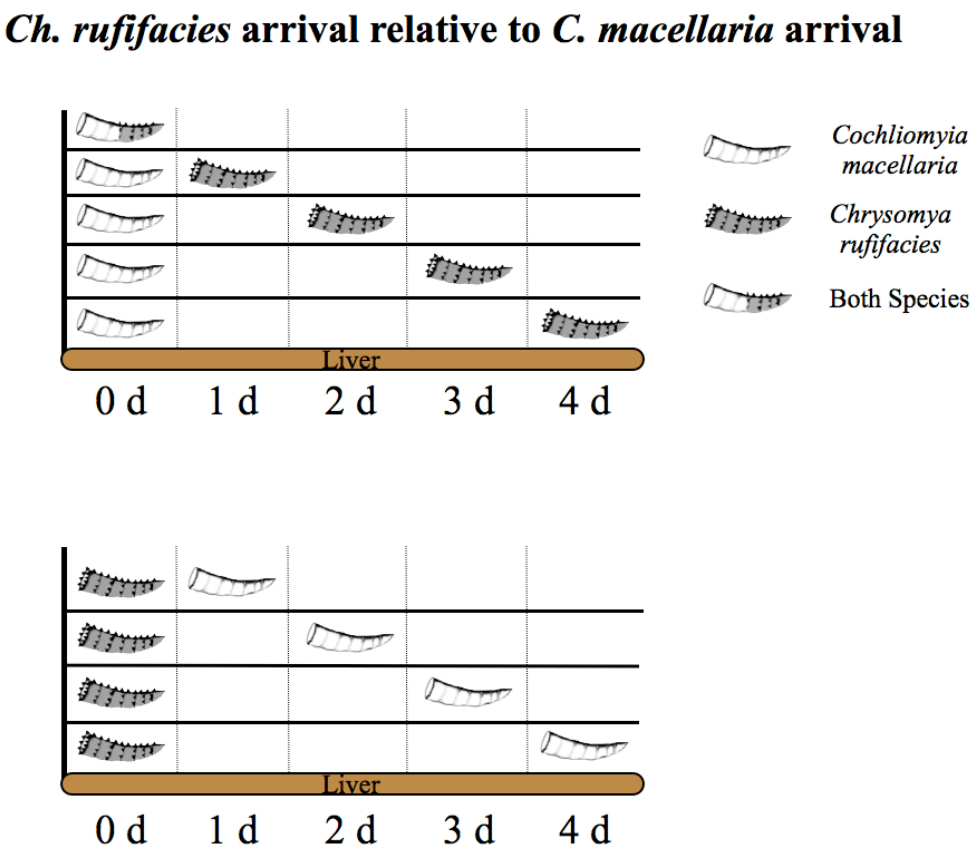


Fig 2.1. Treatments for mixed species experiments. Pioneer species was placed on fresh (0 day old) liver, while competing species was placed simultaneously, or one day, two days, three days, or four days after pioneer species

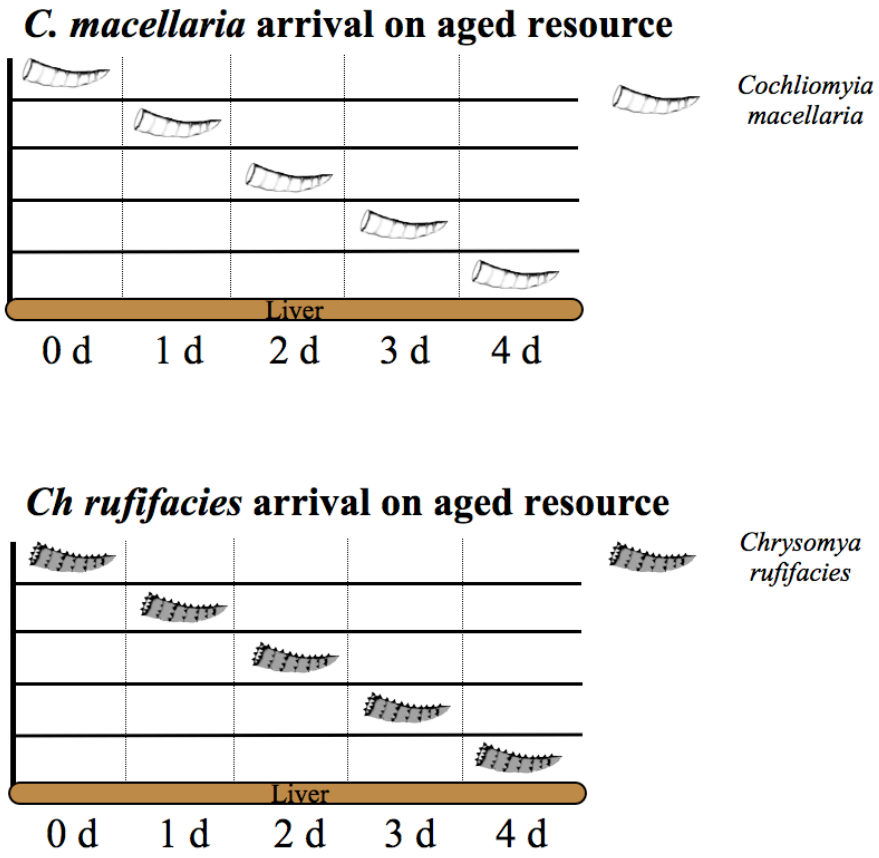


Fig 2.2. Controls for effect of liver age on fitness. Pure cultures of larvae were placed on fresh (0 day old), one day old, two day old, three day old, or four day old liver.

Fitness parameters evaluated for each species included survivorship, pupal weight, adult longevity, and egg production of resulting adults (Allen 2004). Containers were checked every 24 h for pupae, which were then collected from each container and weighed. In order to measure adult longevity, the first 20 pupae observed were placed individually in 30 ml clear plastic cups (Bio-Serv, Frenchtown, NJ, USA) with 5 g autoclaved sand, covered with breathable lids and returned to the growth chamber. Pupae were observed daily for eclosion. Resulting adults were fed 100 µl of 21% concentration sucrose syrup daily via pipette.

Remaining pupae from each treatment and control were placed in 300 cm^3 cages (Bioquip Products, Rancho Dominguez, CA, USA) held in the growth chamber previously described. Resulting adults were provided granulated sugar, water and commercial buttermilk powder *ad libitum*. Colonies were presented with fresh bovine liver for 12 h per d, and resulting eggs were counted. Dead adults were removed from colonies and sexed. Total number of eggs produced by each colony was divided by total number of females present, for a mean number of eggs produced per female.

Stastitical Analysis. All data were analyzed using MANOVA and Tukey's HSD ($P < 0.05$) using IBM SPSS Statistics 18 (SPSS Inc. 2010, Chicago, IL, USA). Data figures were created and managed in GraphPad Prism (GraphPad Software, Inc. La Jolla, CA, USA).

Results

Cochliomyia macellaria and *Ch. rufifacies* development and survivorship were not significantly affected by liver age when reared in pure culture. Survival to pupation for *C. macellaria* ($F = 0.452$; df = 4; $P = 0.769$) and *Ch. rufifacies* ($F = 0.432$; df = 4; $P = 0.793$) were not significantly different due to liver age. Survival to pupation was also not significantly different between species, meaning neither *C. macellaria* nor *Ch. rufifacies* has inherently higher survival rates ($t = 0.742$; df = 34; $P = 0.462$) (Fig. 2.3). Mean pupal weight for *C. macellaria* ($F = 1.412$; df = 4; $P = 0.281$) and *Ch. rufifacies* ($F = 2.535$; df = 4; $P = 0.087$) was also not significantly affected by liver age (Fig. 2.4). *Chrysomya rufifacies* pupae were significantly larger than *C. macellaria* pupae ($t =$

81.22; $P < 0.0001$), outweighing *C. macellaria* by an average 24% (Fig. 2.4). Adult longevity also showed with no significant differences for either *C. macellaria* ($F = 0.2051$; df = 4; $P = 0.0298$) or *Ch. rufifacies* ($F = 0.3349$; df = 4; $P = 0.8484$) (Fig. 2.5). *Cochliomyia macellaria* adults, however, lived significantly longer (34.00 ds vs. 23.54 ds) than *Ch. rufifacies* adults ($t = 3.648$; df = 25; $P = 0.0012$). Neither *C. macellaria* ($F = 0.2051$; df = 4; $P = 0.0298$) or *Ch. rufifacies* ($F = 0.3349$; df = 4; $P = 0.8484$) showed significant differences in fecundity based on resource age; nor was there a significant difference in fecundity between the two species ($t = 0.9768$; df = 28; $P = 0.1940$) (Fig. 2.6).

Both *C. macellaria* and *Ch. rufifacies* fitness were affected by arrival time. *Cochliomyia macellaria* survival to pupation ($F = 5.621$; df = 2; $P = 0.008$) and mean pupal weight ($F = 179.958$; df = 2; $P < 0.0001$) were significantly higher when C. *macellaria* eggs were introduced prior to *Ch. rufifacies* colonization (Fig. 2.7). *Chrysomya rufifacies* had a significantly higher survival to pupation ($F = 7.136$; df = 2; $P = 0.003$), but lower mean pupal weight ($F = 103.091$; df = 2; $P < 0.0001$) when eggs were introduced after *C. macellaria* colonization than when introduced prior (Fig. 2.7). Neither *C. macellaria* ($F = 2.820$; df = 2; $P = 0.062$) nor *Ch. rufifacies* ($F = 2.671$; df = 2; $P = 0.070$) showed significant changes in adult lifespan based on colonization time. *Cochliomyia macellaria* fecundity was significantly reduced when eggs were introduced after *Ch. rufifacies* ($F = 4.393$; df = 2; $P = 0.024$), unlike *Ch. rufifacies* ($F = 1.157$; df = 2; $P = 0.331$) (Fig. 2.7).

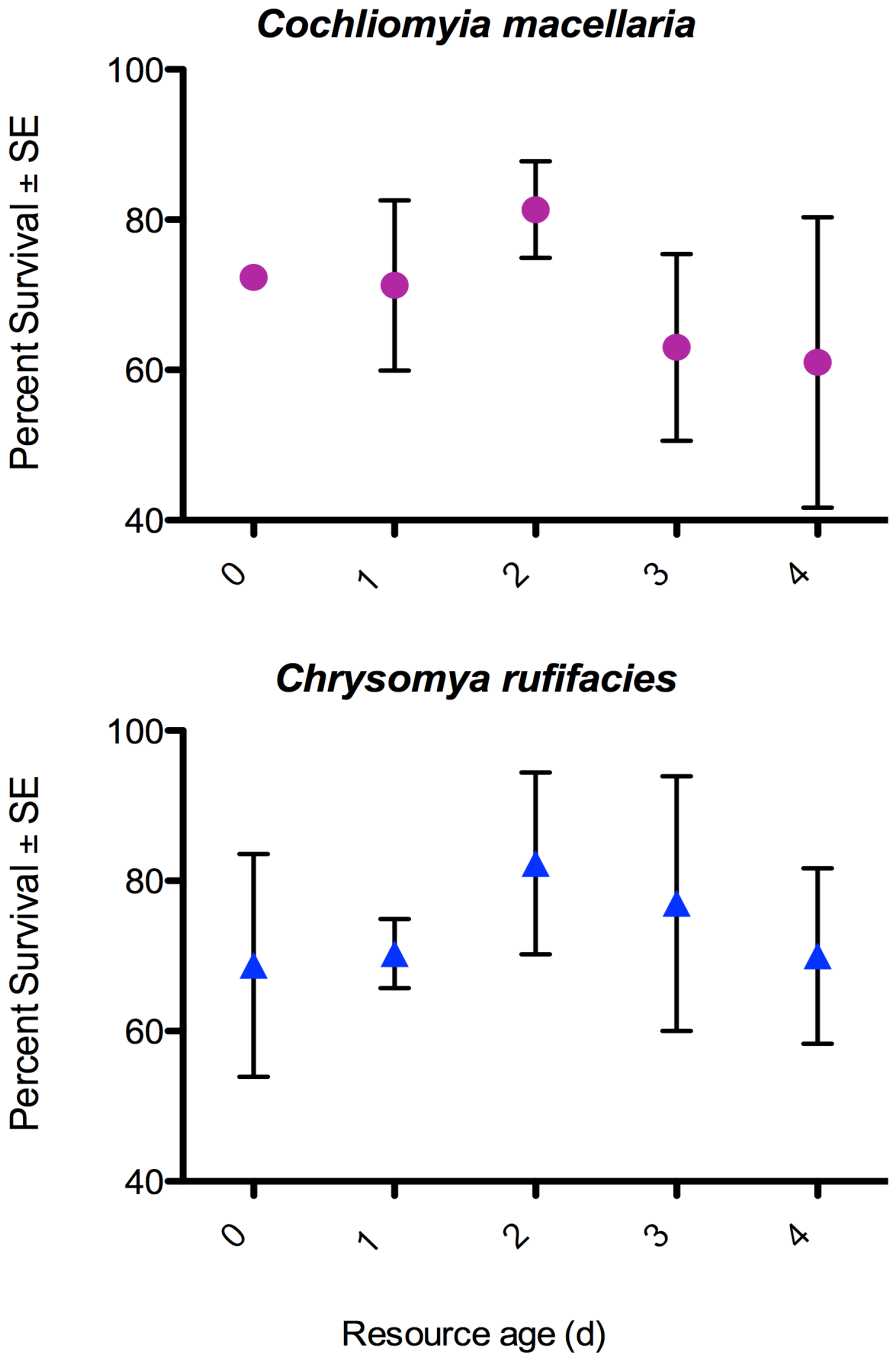


Fig 2.3. Percent larval survival to pupation (mean ± SE) for *C. macellaria* (circle) and *Ch. rufifacies* (triangle) by resource age in days. Resource age did not significantly affect survival to pupation in either species.

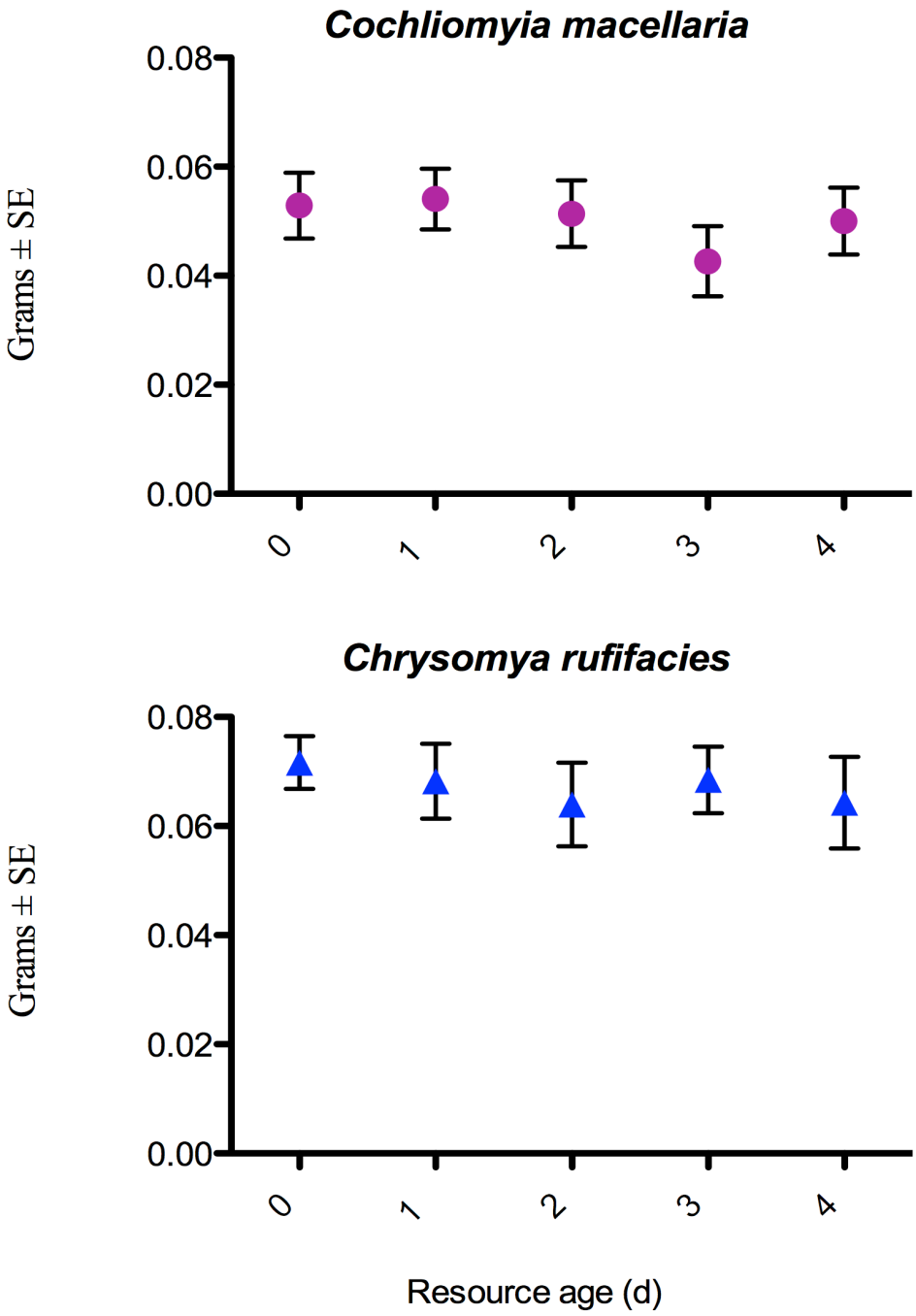


Fig 2.4. Mean pupal weight (mean ± SE) for *C. macellaria* (circle) and *Ch. rufifacies* (triangle) by resource age in days. Resource age did not significantly affect pupal weight in either species.

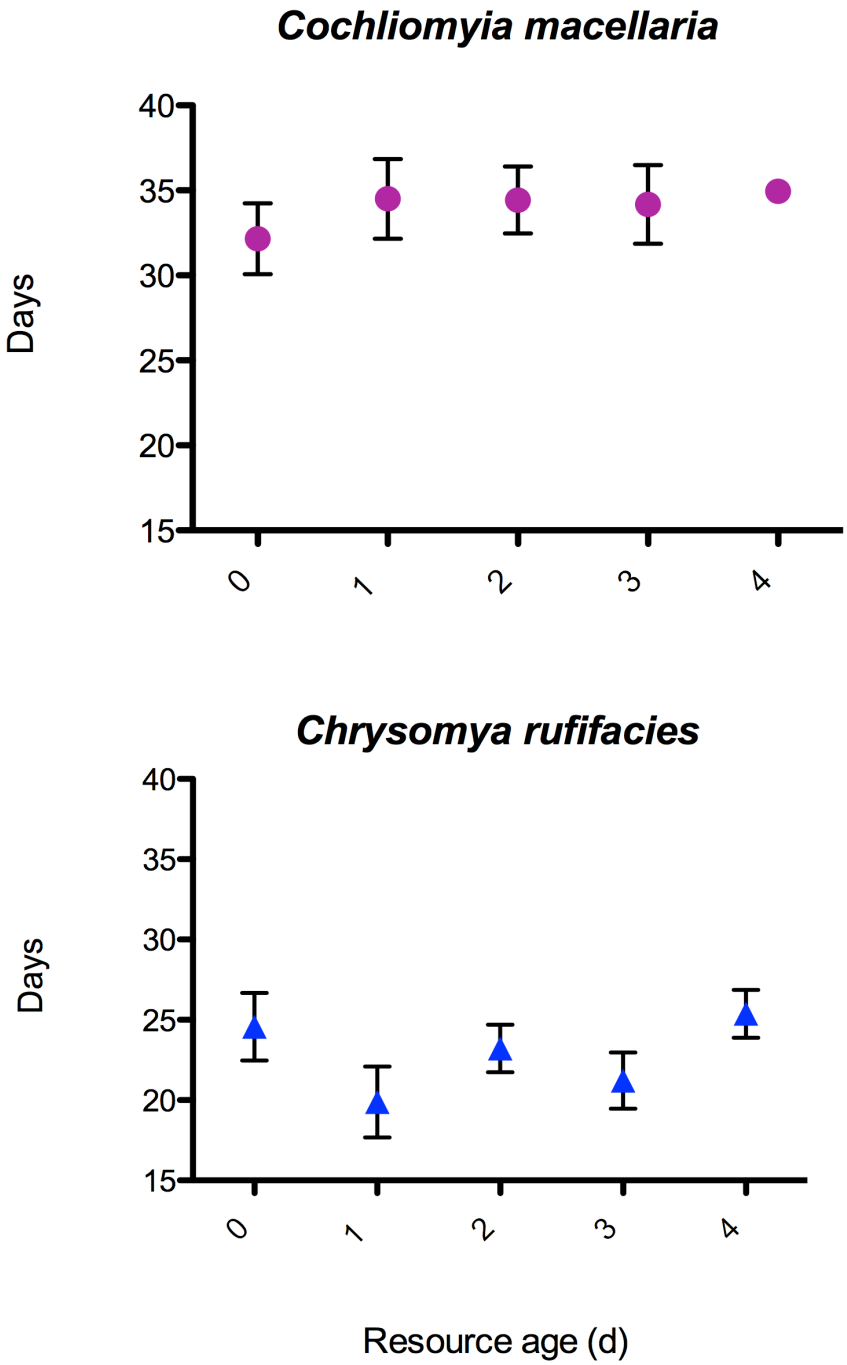


Fig 2.5. Adult longevity in days (mean ± SE) for *C. macellaria* (circle) and *Ch. rufifacies* (triangle) by resource age in days. Resource age did not significantly affect pupal weight in either species.

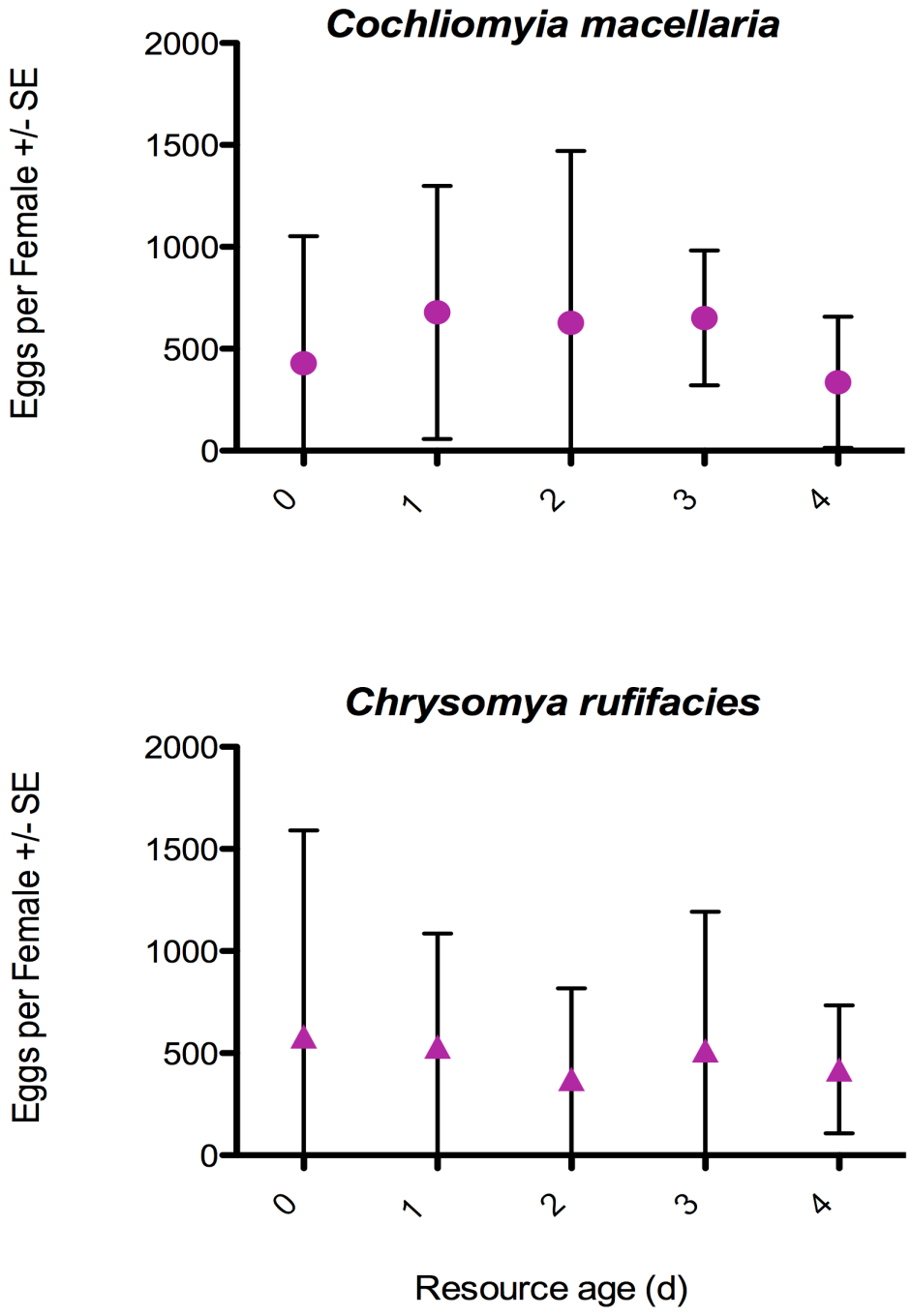


Fig 2.6. Mean eggs per female (mean ± SE) for *C. macellaria* (circle) and *Ch. rufifacies* (triangle) by resource age in days. Resource age did not significantly affect fecundity in either species.

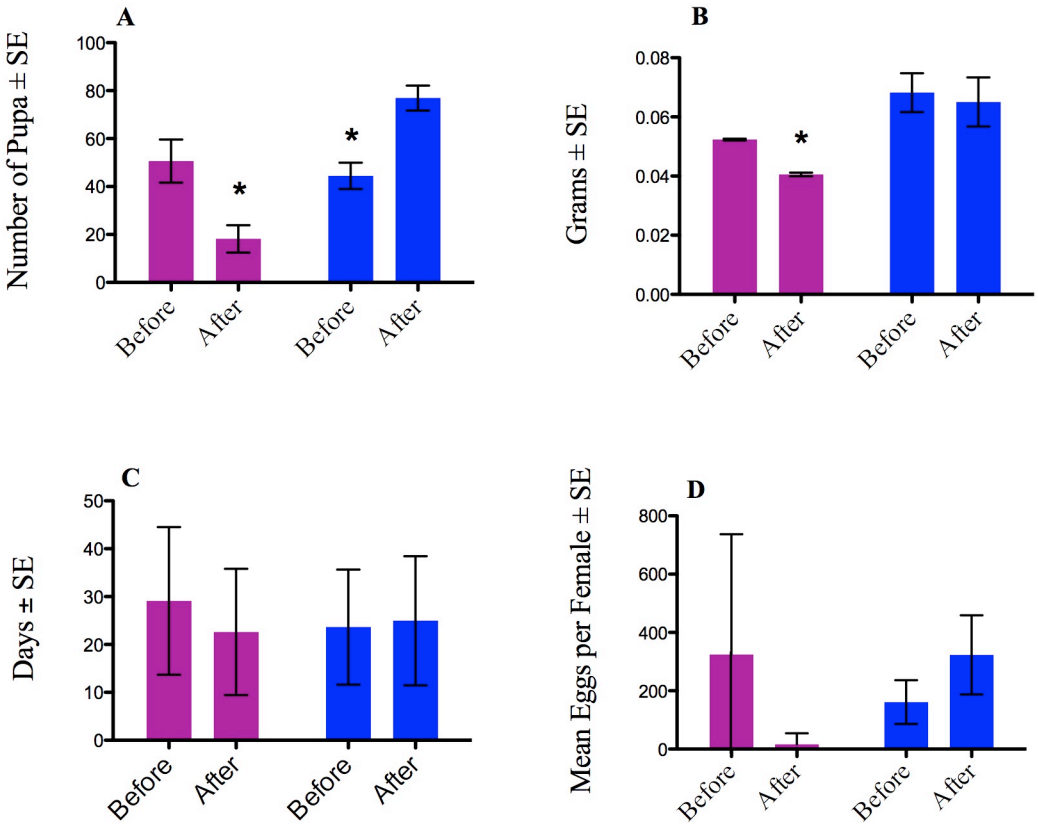


Fig 2.7. Differences in (A) survival, (B) pupal weight, (C) longevity, and (D) fecundity (mean ± SE) for *C. macellaria* (purple) and *Ch. rufifacies* (blue) according to arrival time. Species arrived either before or after competing species. * indicates significant ($P \leq 0.05$) differences between means.

Cochliomyia macellaria survivorship, pupal weight, and fecundity were affected by when *Ch. rufifacies* colonized the resource ($F = 1652.090$; df = 8; $P < 0.0001$). *Cochliomyia macellaria* showed greater fitness when it was not sharing the resource with *Ch. rufifacies* larvae of similar age. When *C. macellaria* was the initial colonizer, its survival to pupation was not impacted when *Ch. rufifacies* arrived 4 d prior to (M = 48.67; SD = 8.505), or 3 d (M = 66.00; SD = 0.00) or 4 d after (M = 87.5; SD = 17.68) it. However, *Ch. rufifacies* arrival 3 d (M = *40.67; SD* = 18.77), 2 d (M = 0.25; SD = 0.50),

or 1 d (*M* = 3.25; *SD* = 3.948) prior to *C. macellaria* colonization resulted in significant decrease in *C. macellaria* survival. When *Ch. rufifacies* arrived simultaneously with (*M*=25.5; *SD*=9.678), 1 d (*M*=22.0; *SD*=14.73), or 2 d (*M*=29.33*; SD*=25.42) after *C. macellaria* colonization decreased *C. macellaria* survivorship (Fig. 2.8).

Chrysomya rufifacies responded differently to colonization time. It showed significantly greater fitness when it did share the resource with *C. macellaria* larvae of similar age. Ch*. rufifacies* arriving 4 d prior to *C. macellaria* had significantly lower survival to pupation (*M* = 35.0; *SD* = 5.88). *Chrysomya rufifacies* arriving 1 d (*M* = 86.75; *SD* = 9.069) after *C. macellaria* colonization had significantly higher survival rates than the corresponding control (*M* = 70.33; *SD* = 4.619). *Chrysomya rufifacies* exhibited its highest survivorship when it arrived 1 to 3 d after *C. macellaria* colonization (Fig. 2.9). All other arrival times did not significantly impact *Ch. rufifacies* survivorship (Fig. 2.9).

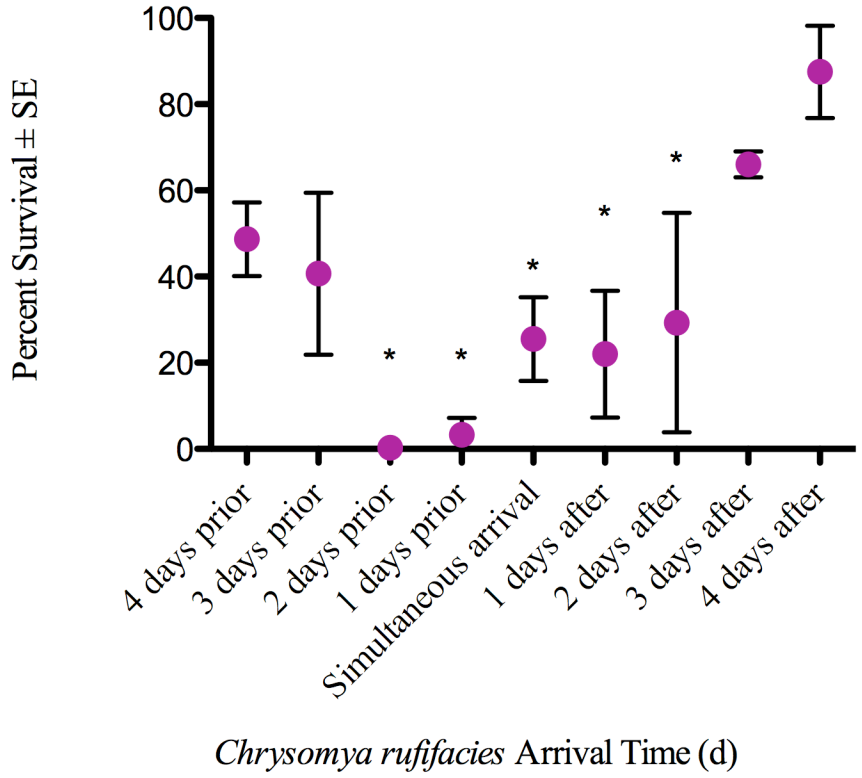


Fig 2.8. *Cochliomyia macellaria* survival to pupation (mean ± SE) relative to *Ch. rufifacies* colonization. * indicates significant ($P \leq 0.05$) difference from control.

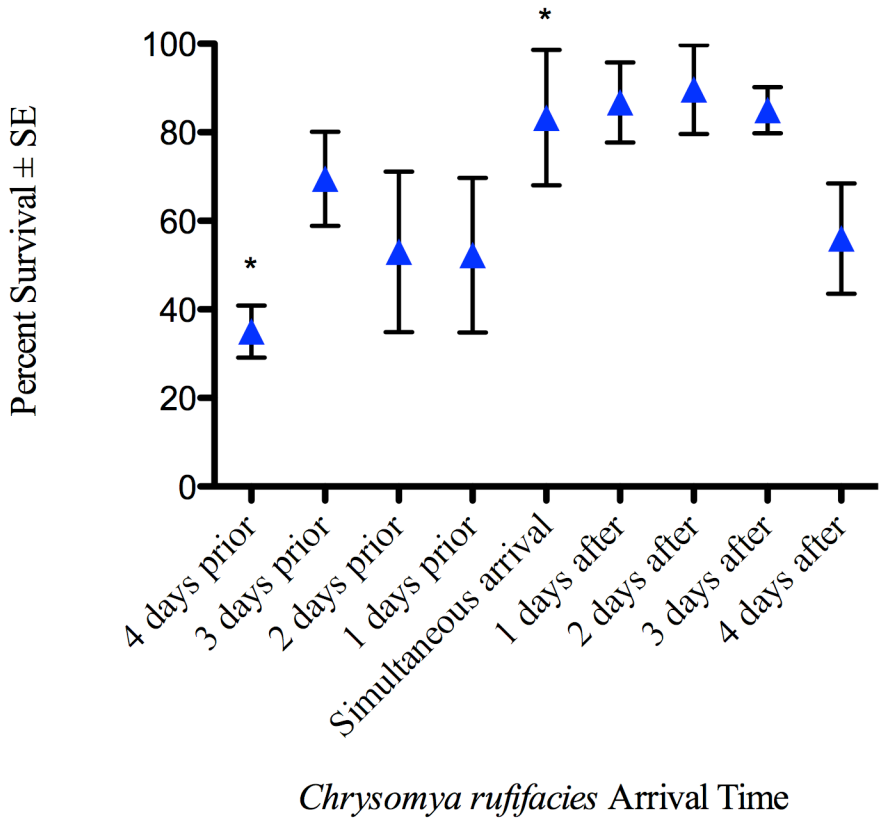


Fig 2.9. *Chrysomya rufifacies* survival to pupation (mean ± SE) relative to *Ch. rufifacies* colonization. * indicates significant ($P \leq 0.05$) difference from control.

Pupal weight mirrored larval survivorship in *C. macellaria*. Significant differences in pupal weight due to *Ch. rufifacies* arrival time ($F = 1652.090$; df = 8; $P < 0.0001$) were detected. *Ch. rufifacies* arriving 4 d ($M = 0.043$; SD = 0.005), 3 d ($M = 0.029$; $SD = 0.005$), 2 d ($M = 0.012$; $SD = 0.005$), or 1 d ($M = 0.034$; $SD = 0.012$) prior to, or simultaneously with ($M = 0.046$; $SD = 0.006$) *C. macellaria*, resulted in lower *C. macellaria* pupal weight than those from the controls. *Chrysomya rufifacies* arrival 1 d ($M = 0.049$; $SD = 0.005$), 2 d ($M = 0.054$; $SD = 0.0085$), 3 d ($M = 0.049$; $SD = 0.008$), and 4 d ($M = 0.049$; $SD = 0.0072$) after *C. macellaria* colonization did not result in

lower *C. macellaria* pupal weights (Fig. 2.10). *Chrysomya rufifacies* again showed very different results, only exhibiting significantly lower pupal weights when arriving 3 d after *C. macellaria* ($M = 0.05810$; $SD = 0.006601$) (Fig. 2.11).

Neither *C. macellaria* nor *Ch. rufifacies* showed significant differences in adult longevity due to *Ch. rufifacies* colonization time (Figs. 2.12 and 2.13). The only significant difference between treatments and controls was for *C. macellaria* pupae resulting from larvae that colonized 1 or 2 d after *Ch. rufifacies* did not eclose. This resulted in no adults for any of the treatments, and therefore a mean adult longevity of 0 d (Fig. 2.12).

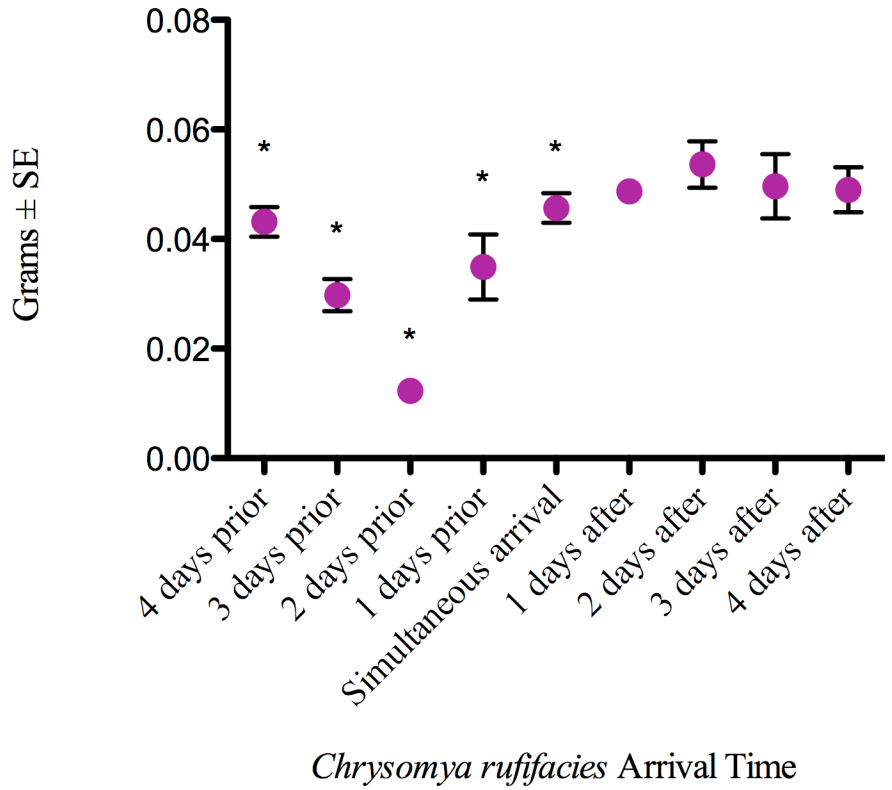


Fig 2.10. *Cochliomyia macellaria* pupal weight (mean ± SE) relative to *Ch. rufifacies* colonization. * indicates significant ($P \leq 0.05$) difference from control.

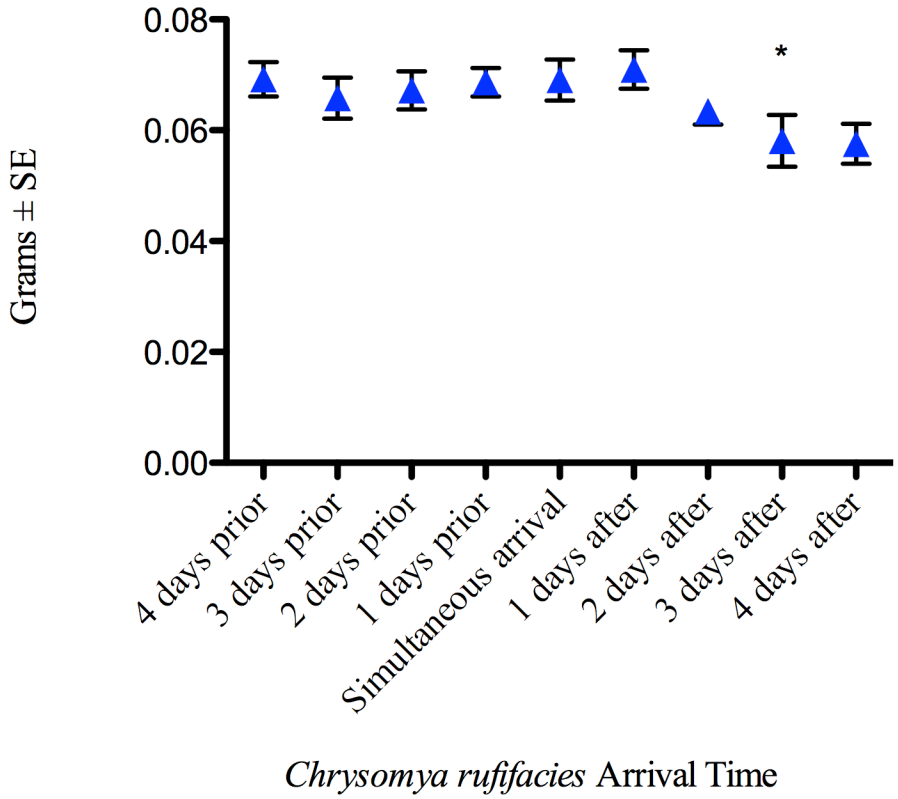


Fig 2.11. *Chrysomya rufifacies* pupal weight (mean ± SE) relative to *Ch. rufifacies* colonization. * indicates significant ($P \leq 0.05$) difference from control.

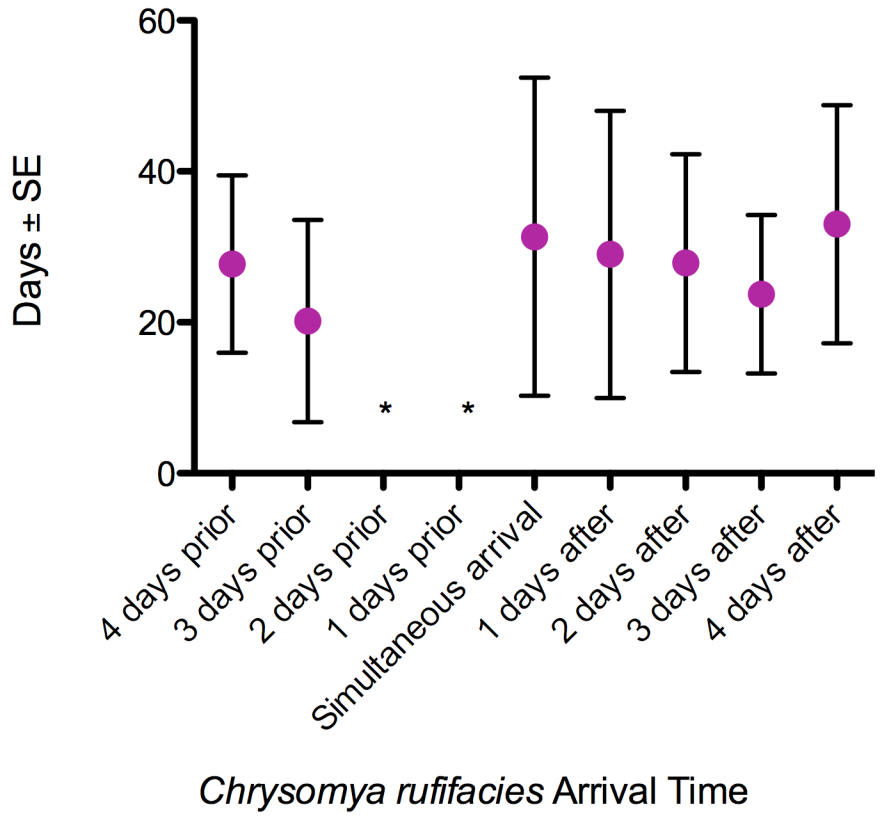


Fig 2.12. *Cochliomyia macellaria* adult lifespan (mean ± SE) relative to *Ch. rufifacies* colonization. * indicates significant ($P \leq 0.05$) difference from control.

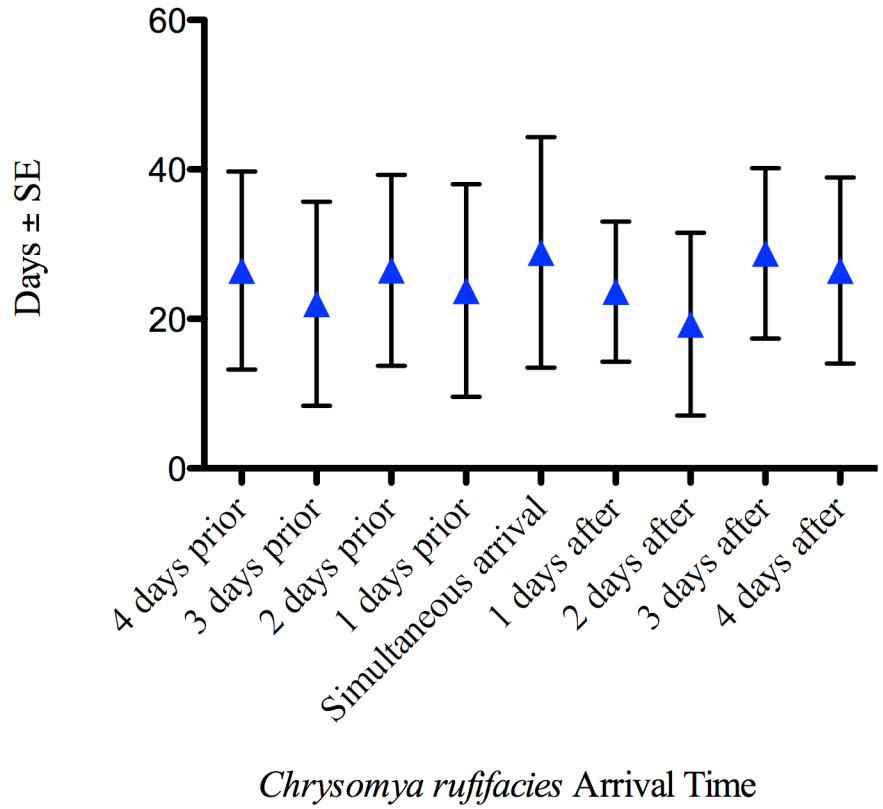


Fig 2.13. *Chrysomya rufifacies* adult lifespan (mean ± SE) relative to *Ch. rufifacies* colonization. * indicates significant ($P \leq 0.05$) difference from control.

Egg production per female varied among treatments, but followed similar patterns to survivorship and pupal weight. *Cochliomyia macellaria* arriving 4 d (*M* =3 59.5; *SD* = 139.5), 3 d (*M* = 290.7; *SD* = 503.4), 2 d (*M* = 262.8; *SD*=455.3) and 1 d (*M* = 3.84; *SD*=665.8) prior to *Ch. rufifacies* produced significantly fewer eggs per female than the corresponding controls. Those *C. macellaria* that arrived simultaneously with or after *Ch. rufifacies* had no significant difference in mean egg production when compared to controls (Fig. 2.14). *Chrysomya rufifacies*, however, had no significant difference in mean egg production per female when compared to controls (Fig. 2.15).

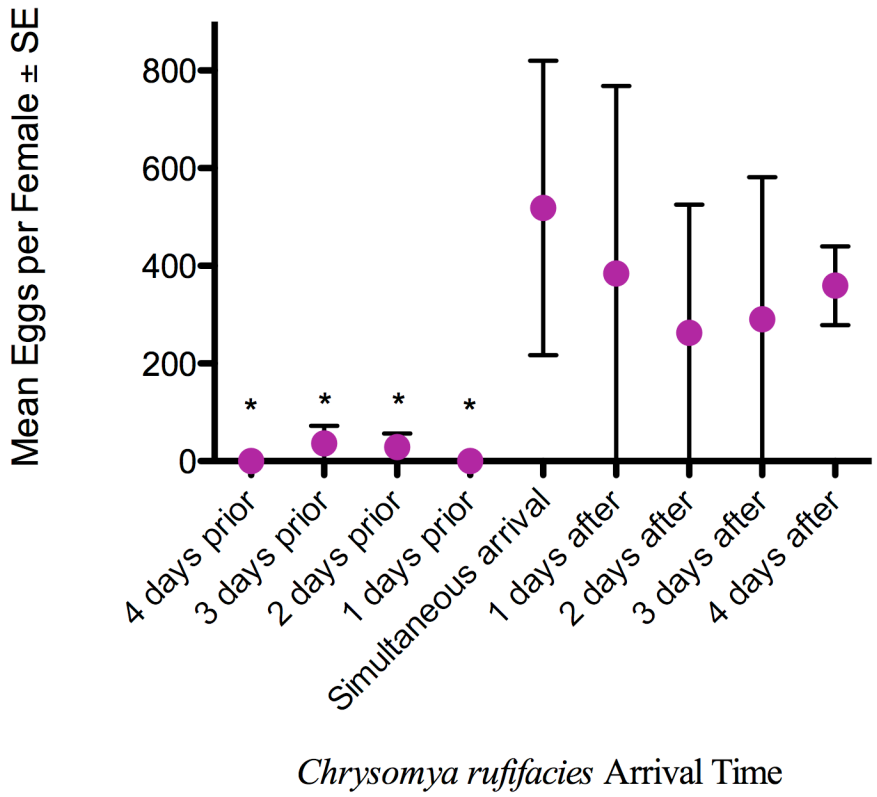


Fig 2.14. *Cochliomyia macellaria* eggs per female (mean ± SE) relative to *Ch. rufifacies* colonization. * indicates significant ($P \leq 0.05$) difference from control.

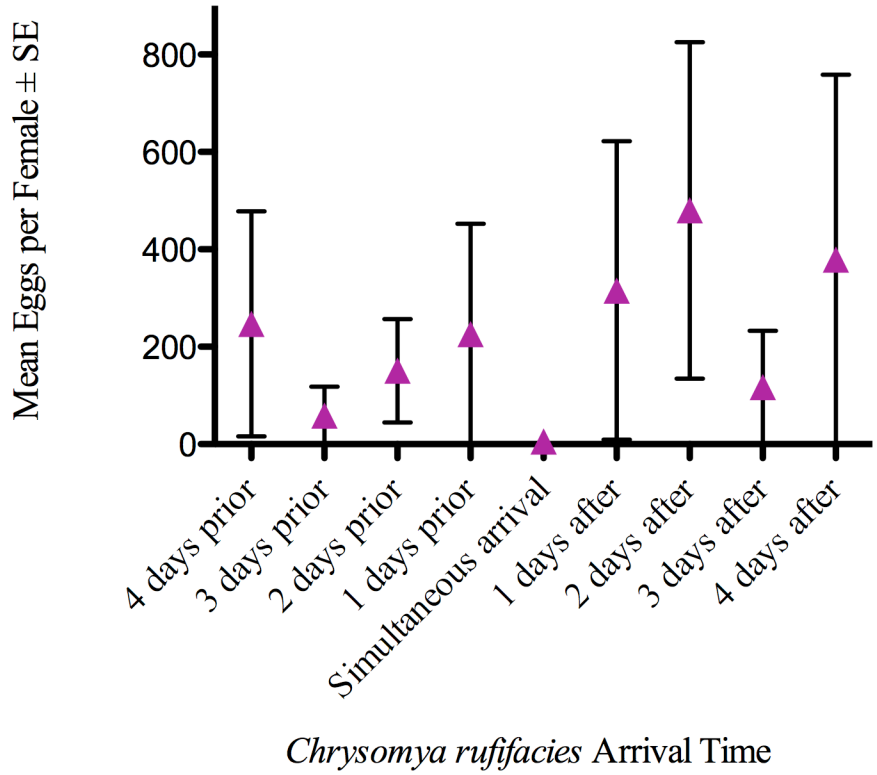


Fig 2.15. *Chrysomya rufifacies* eggs per female (mean ± SE) relative to *Ch. rufifacies* colonization. * indicates significant ($P \leq 0.05$) difference from control.

Discussion

Results from this study demonstrate a priority effect between *Ch. rufifacies* and *C. macellaria*, and imply that the predator-prey relationship between the two species may be more important than competition for food. Priority effect is known in Calliphoridae, as Hanski (1987) determined that carrion flies arriving first at a carcass dominated the resource, effectively preventing further colonization by later species (Hanski 1987). Kneidel (1983) also demonstrated priority effects in carrion-breeding Diptera, finding that *Megaselia scalaris* (Loew) (Diptera: Phoridae) reduced secondary

colonizer diversity when it acted as a primary colonizer (Kneidel 1983). Schoenly and Reid (1987) postulated facilitation of arthropod community change depended heavily upon primary colonizers, which altered the resource allowing location (Spivak et al. 1991) and colonization by secondary species (Schoenly and Reid 1987). This implies that secondary species rely on the presence of primary colonizers to prepare the resource for their colonization resulting in higher fitness with increased survivorship of associated offspring. This study illustrated this concept utilizing the predator-prey model of *Ch. rufifacies* and *C. macellaria*.

Ch. rufifacies has historically been considered a primary necrophage and a facultative predator (Goodbrod 1990, Baumgartner 1993, Rosa et al. 2006). It was assumed that predation began only when resource was scarce (Rosa et al. 2006), allowing *Ch. rufifacies* to survive even as a secondary colonizer on a depleted resource (Faria 2004). Results from this study, however, indicate otherwise. *Chrysomya rufifacies* larvae that were present on the resource concurrently with *C. macellaria* larvae had a significantly higher fitness than those that were on the resource before or after (Fig. 2.7). This is unexpected if it is assumed that *Ch. rufifacies* maintains secondary colonization status to avoid competition with primary colonizers. An inferior colonizer would show substantially decreased fitness when forced to directly compete with equivalent species (Hanski 1987). Since this was not the case, it appears that *Ch. rufifacies* is not delaying colonization to avoid competition.

A second possibility for maintaining secondary colonizer status is the modification of resource by primary colonizers. The community of arthropods inhabiting

carrion early will change the resource (Schoenly and Reid 1987, Spivak et al. 1991), which may allow secondary colonizers to use the resource more efficiently. However, this experiment showed that *Ch. rufifacies* shows equal fitness on fresh and aged resource (Fig. 2.3 – 2.6). If *Ch. rufifacies* required modification of the resource to enable efficient nutrient intake, fresh resource should have caused a decline in fitness. Since this was not the case, it appears that resource modification is not the selective force behind *Ch. rufifacies* secondary colonization.

While resource consumption was not directly measured, observations of the resource before and after colonization of both species showed that *C. macellaria* consumed most of its food resource prior to pupation. *Chrysomya rufifacies,* on the other hand, left substantial resource behind, yet still were able to successfully pupate and eclose. This behavior was observed even in pure cultures of *Ch. rufifacies* and those mixed with *C. macellaria*. This difference in resource consumption, along with its maintenance as a secondary colonizer despite apparent selection against such behavior, implies that *Ch. rufifacies* is a facultative necrophage and a primary predator.

The presence of *Ch. rufifacies* and not resource age (Fig. 2.4) impacted *C. macellaria* fitness (i.e., egg production/female). *Cochliomyia macellaria* was most fit when arriving 4 d before or after *Ch. rufifacies* (Fig. 2.7). Arriving 4 d prior allowed *C. macellaria* adequate time to reach the pupal stage prior to the onset of competition or potential predation by *Ch. rufifacies*. Arriving 4 d after allowed *C. macellaria* to inhabit the resource after *Ch. rufifacies* pupated. Either time frame enabled *C. macellaria* to inhabit “enemy-free” space with reduced competition for long enough to complete

development without compromising survivorship, pupal weight, longevity or fecundity. Close association with *Ch. rufifacies* was directly correlated to fitness decline in *C. macellaria*. *Chrysomya rufifacies* potentially began predation while *C. macellaria* larvae were still present forcing them to leave the resource before reaching the minimum viable weight necessary to successfully pupate. *Cochliomyia macellaria* larvae arriving within 2 d after *Ch. rufifacies* exhibited the most dramatic decrease in survivorship, pupal weight, and adult longevity of all the treatments. The 2 d lead time allowed *Ch. rufifacies* to reach the predaceous second and third instars prior to *C. macellaria* oviposition (Wells and Greenberg 1992a, Byrd and Butler 1996, Sukontason et al. 2004). This resulted in a 98% reduction of *C. macellaria* on the resource; those few larvae that were able to make it to pupation were undersized in comparison to those from the controls and did not eclose (Fig. 2.5).

Chrysomya rufifacies fitness was also impacted by the presence of *C. macellaria*. *Chrysomya rufifacies* colonizing 4 d prior to *C. macellaria* exhibited its lowest (35%) survivorship. This may be due to two reasons. First, the 4 d lead time allowed *Ch. rufifacies* to complete its larval stages before *C. macellaria* entered the patch, thus eliminating opportunities to predate on *C. macellaria* larvae. This temporal delay could increase the risk of cannibalism due to the lack of prey items (Goodbrod 1990) and explain the significantly lower survival rates. Second, first instar *Ch. rufifacies* are known to join interspecific larval masses (Baumgartner 1993). Since first instar *Ch. rufifacies* are unable to predate other larvae, this interspecific aggregation may facilitate efficient feeding on the resource, a hallmark of interspecific larval masses (Rivers et al.

2011). *Chrysomya rufifacies* colonization delay may have kept it from exploiting the interspecific maggot mass, thereby lowering feeding efficiency. This delay again raised the risk of *Ch. rufifacies* cannibalism and decreased survivorship.

Fitness reduction in *C. macellaria* may also be attributed to non-consumptive effects induced by *Ch. rufifacies.* Predators influence prey populations by both directly consuming individuals (consumptive effects), and by altering prey behavior (non-consumptive effects) (Peckarsky et al. 2008, Sih et al. 2010). Non-consumptive effects may impact prey physiology and in the long run have a greater consequence than being directly consumed (Dill et al. 2003). For example, *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) exhibit a 25% decrease in developmental time when in the presence of *Phytoseiulus persimilis* (Athias-Henroit) (Acari: Phytoseiidae), a predatory mite, resulting in lower feeding rates, and smaller resulting adults (Walzer and Schausberger 2009) which could translate into fewer offspring produced. Cues released by predatory *Ch. rufifacies* could result in accelerated development of their larval prey (Aguiar-Coelho and Milward-de-Azevedo 1998) resulting in smaller adults and thus reducing *C. macellaria* adult size and fitness. Because *Ch. rufifacies* is predaceous in the second and third instars (Wells and Greenberg 1992a), early-colonizing *C. macellaria* only has a limited amount of time to reach critical weight before being potentially consumed. Those taking longer to reach the pre-pupal stage may be consumed, while those that exhibit shorter developmental times may have a greater chance of reaching adulthood. This process could cause a developmental shift in *C. macellaria* populations

that coexist with *Ch. rufifacies*, resulting in a significantly faster *C. macellaria* developmental time.

Deciphering the ecological interactions between *C. macellaria* and *Ch. rufifacies* allows greater understanding of the impact of an introduced blow fly species on native blow flies in the Western Hemisphere, and the corresponding decomposition ecology of carrion. These data are also important for forensic entomology. Estimates of the age of a carrion resource are extrapolated from estimates of the development time of the larvae collected from remains (Pruzan and Bush 1977) (Greenberg 1991). This method depends upon extensive knowledge of both the community of decomposers and the larval developmental rates of forensically important flies. A change in either of these parameters could result in inaccurate estimates of the period of insect activity (PIA) as related to the minimum postmortem interval (m-PMI).

Cochliomyia macellaria is considered a primary colonizer of carrion and is therefore extensively used in forensic entomology in the US (Tenorio et al. 2003, Oliveira-Costa and Mello-Patiu 2004, Gomes et al. 2009). Life-history tables used to estimate larval age have been developed based on *C. macellaria* populations either naive to *Ch. rufifacies* predation (Byrd and Butler 1996), or not (Kirkpatrick and Olson 2007, Boatright and Tomberlin 2010). I have shown that *C. macellaria* development varies due to the presence or absence of *Ch. rufifacies*, which can result in error when estimating the PIA or m-PMI of the remains from which they were collected. Developmental data obtained from *C. macellaria* larvae reared without *Ch. rufifacies* may therefore be inaccurate when applied to cases in which both species occur.

In addition to the impact of *Ch. rufifacies* on *C. macellaria*, forensic entomologists need to understand the fitness impacts of delayed colonization incurred by *Ch. rufifacies*. Currently, larvae found inhabiting carrion are assumed to have arrived soon after death of the resource. However, the increased survivorship, longevity, and fecundity received by *Ch. rufifacies* colonizing 1 to 2 d after resource death and resulting predation of primary colonizers selects for delayed colonization. Given the ability of this species to predate upon other larvae in the maggot mass, it is a possibility that *Ch. rufifacies* may be the only species available for use during an investigation. Therefore, forensic entomologists should take into account the possibility of delayed oviposition and colonization by extending time of colonization estimations by 1 to 2 d.

The obvious impact of arrival time on these two species of blow fly implies a mechanism of colonization regulation. Each species must have the ability to recognize a suitable resource, and colonize such a resource efficiently. An understanding of the mechanisms used by each species to identify proper colonization windows would help clarify successional patters in carrion insects, and allow for a deeper understanding of decomposition ecology.

CHAPTER III

ATTRACTION OF *COCHLIOMYIA MACELLARIA* AND *CHRYSOMYA RUFIFACIES* TO FRESH AND PUTRID LIVER IN A DUAL CHOICE OLFACTOMETER

Introduction

Putrefaction of vertebrate remains is initiated at death (Campobasso 2001, Carvalho et al. 2001, Dekeirsschieter et al. 2010). This process results in the productions of volatile organic compounds (VOC) (Archer and Elgar 2003) that attract blow flies (Diptera: Calliphoridae) that feed on, and colonize, the resource (Browne 1960, Wallis 1962). Concentration and makeup of these VOC influence blow fly attraction (Ashworth and Wall 1994). Arthropod species arrival and colonization patterns are likely regulated partially by VOC emissions (von Hoermann et al. 2011).

VOC concentrations change as carrion decomposes. Early decomposition is characterized by the emission of ethanol, 2-propanone, dimethyl disulfide, and methyl benzene (Statheropoulos et al. 2007), while advanced decomposition is characterized by dimethyl disulfide, toluene, hexane, and benzene (Statheropoulos et al. 2005). These substances are responsible for the characteristic odor of decomposition (VOC) and attract differing arthropods depending on concentration and mixture (von Hoermann et al. 2011).

Behavioral responses of calliphorids to these VOC are regulated by their physiological (Browne 1993) status. Oviposition behavior by *Calliphora vomitoria*

(Linnaeus) (Diptera: Calliphoridae) is not exhibited by females with underdeveloped ovaries (Browne 1993). Ashworth and Wall (1995) found that *Lucilia sericata* with completely developed ovarioles increased searching activity when presented with liver odors (Ashworth and Wall 1995). Females that did not have fully developed ovarioles showed periods of suppressed activity, even when presented with high concentrations of liver odors (Ashworth and Wall 1995). Similarly, Wall (1994) found that gravid *L. sericata*, in comparison to those with partially developed ovaries, increased both the number and duration of flights towards carrion odor by 50%

Protein depravation affects blow fly attraction to a resource (Ashworth and Wall 1995, Aak et al. 2010). Differences in physiological state due to such deprevation may alter the response to olfactory cues (Ashworth and Wall 1995). For example, consumption of protein-rich material by anautogenous flies such as *Phormia regina* (Dethier 1961), *Musca autumnalis* (De Geer) (Diptera: Muscidae) (Van Geem and Broce 1986), and *Lucilia cuprina* (Wiedemann) (Roberts 1974), declines as oocytes mature, yet gravid *L. sericata* showed a 60% increase in searching activity over non gravid flies when presented with liver odors (Ashworth and Wall 1995).

Male adult blow flies have differing protein requirements from females, and therefore may not need to use decomposing resources as a feeding site. *Lucilia cuprina* males intake 50% less protein over their life time than females of the same species (Roberts 1974). Experiments with *P. regina* indicate that males are able to ingest enough protein by feeding on the fecal specs of protein-fed females (Dethier 1961). Males may therefore not respond to protein-derived odors in the same way as females.

Cochliomyia macellaria (Fabricius) and *Chrysomya rufifacies* (Macquart) (Diptera: Calliphoridae) are common blow flies in the southern US and are associated with carrion. However, their arrival and colonization patterns on carrion are quite different (Fuller 1934b, Early and Goff 1986, Eberhardt and Elliot 2008, Biavati et al. 2010). *Cochliomyia macellaria* acts as a primary colonizer, arriving at the carrion source within 24 h after its death (Ortiz and Tomberlin 2009), while *Ch. rufifacies* acts as a secondary colonizer arriving 24-48 h after death of the carrion (Fuller 1934b, Early and Goff 1986, Eberhardt and Elliot 2008, Cammack and Nelder 2010). *Chrysomya rufifacies* also is a facultative predator, feeding on only the carrion source and other larvae present (Baumgartner 1993, Faria 2004). Variation in arrival and colonization pattern of carrion indicates they utilize different, or variations within, sets of cues.

The mechanisms governing *C. macellaria* and *Ch. rufifacies* arrival patterns are undetermined. These cues may be visual (Wallis 1962, Easton and Feir 1991, Collins 1996, Gomes et al. 2007), auditory (Wertheim et al. 2005, Wicker-Thomas 2007), tactile (Eismann 1988, Easton and Feir 1991), or olfactory (Crombie 1944, Browne 1960, Gomes et al. 2007), or dependent on cues released by interspecifics or conspecifics inhabiting the resource (Judd 1992, Davies 1998, Diaz-Fleischer and Aluja 2003). I investigated the behavioral responses of *C. macellaria* and *Ch. rufifacies* of both sexes as related to their physiological states to resource varying in age in a Y-tube olfactometer to determine if VOCs from the resource served as a mechanism governing their response.

Materials and Methods

Adult Fly Colony. Laboratory colonies of *C. macellaria* and *Ch. rufifacies* were initiated from specimens collected in Brazos County, Texas, USA during spring and summer of 2009 and 2010. Larvae were reared at a standard density of 100 larvae on 50 g of fresh bovine liver in 3-L plastic containers. All rearing was done in a Rheem Environmental in walk-in growth chamber (Ashville, NC, USA) at 27°C ± 1°C, 60% RH, and a 12:12 (L:D) photoperiod. Dispersing third instar larvae were transferred to 3-L containers with autoclaved sand (Town & Country Landscape Supply Co., Chicago, IL, USA) for pupation. Resulting adults were maintained in 300 cm^3 cages (Bioquip Products, Rancho Dominguez, CA, USA) held in the growth chamber previously described. Granulated sugar (Imperial Sugar Co., Sugar Land, TX, USA), buttermilk powder (Saco foods Inc., Middleton, WI, USA), and water were provided *ad libitum*, and 20 g bovine liver was placed in the cage between 2 and 5 d post emergence for 8 h to induce ovarian development.

Behavioral Bioassay. A dual choice olfactometer was used to evaluate the choice of calliphorid adults to fresh or aged resource. The olfactometer was comprised of three stacked 2.7 cm thick Teflon® sheets, covered by a removable sheet of glass (Fig. 3.1).

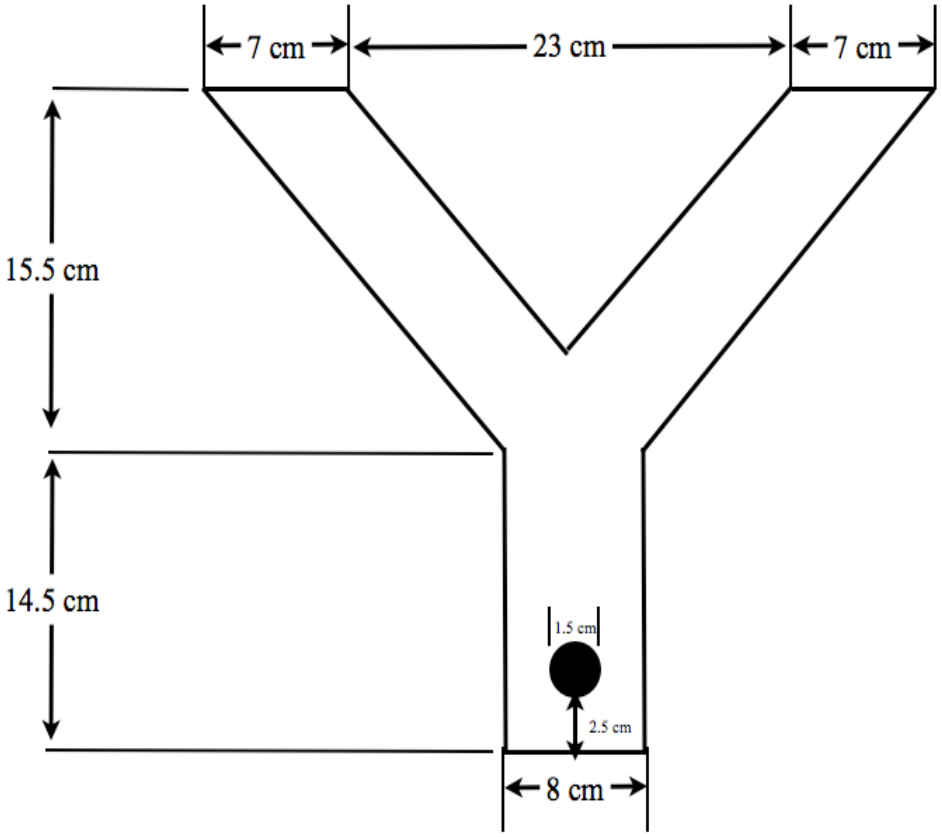


Fig 3.1. Schematic of the dual choice olfactometer indicating size in centimeters.

An intake port measuring 1.3 cm in diameter was located on the terminal end of each arm. An exhaust port measuring 1.7 cm in diameter was located at the terminal end of the stem. An additional 1.6 cm access port for introducing the flies to the olfactometer was located in the base of the olfactometer stem approximately 2.5 cm from the terminal end. Air was pulled through the olfactometer with a 50 mm USB powered computer cooling fan (5VDC Fan, Dc Fans, Thermal Management NMB Technologies Corporation, Chatsworth, CA, USA) mounted to the external surface of the outflow port at the terminal end of the stem. Airflow through the olfactometer at the access port was

measured using an anemometer (Testo 435-1, Testo, Inc., Sparta, NJ, USA) and determined to be 0.5 m/sec. Preliminary runs sans resource were conducted with colony flies to ensure adequate wind speed and assure lighting, temperature, and ambient odors did not induce behavioral responses. These olfactometer integrity checks were replicated every three weeks throughout the experiment.

Air pulled into the olfactometer was cleaned prior to entry by passing it through 15 mm diameter, 14.5 cm long glass tubes containing activated charcoal (Aqua-Tech, Marineland Aquarium Products, Moorpark, CA, USA) and plugged with polyester filter floss (Aqua-Tech, Marineland Aquarium Products, Moorpark, CA, USA). These columns were attached to 15 cm x 15 cm x 12 cm plastic chambers (S.C. Johnson & Son, Inc., Racine, WI, USA), which held the treatments. Chambers were attached to olfactometer arms with 7 cm Teflon® tubing, and chambers were replaced after the completion of each experiment. Chambers were assigned and used with a single treatment. Two fluorescent light tubes (60 W) served for overhead illumination. Temperature in the olfactometer room was approximately 24.0°C ± 2.0°C.

Experimental Design. Approximately 5 g bovine liver was used as the treatment, as this amount is appropriate to stimulate oviposition and feeding by Calliphoridae (Ashworth and Wall 1995).

Resource stock was kept at -20°C. Fresh resource was thawed to room temperature and used within 12 h of removal from freezer. Aged resource, which was taken from the same source as the fresh liver, was thawed to room temperature and incubated in a Rheem Environmental in walk-in growth chamber at 37°C for 24 h to

induce putrefaction.

Behavioral response of *C. macellaria* and *C. rufifacies* to the two resources described was measured. Individual male, gravid and non-gravid females approximately 7-d-old were examined for their response to the following paired treatments; a) fresh liver and a blank resource chamber (determine attraction and repellency); b) putrefied liver and a blank resource chamber (determine attraction and repellency); and c) fresh liver and putrefied liver (determine preference).

Flies were collected from colony cages and placed individually in glass vials (2-dram, 40 mm height x 17 mm diameter) for sexing prior to experiments. Flies were allowed to acclimate for approximately 30 min before their use in the olfactometer. The olfactometer was cleaned with 80% ethanol and allowed to air dry for 2 min before conducting an experiment. Treatment location in the olfactometer arms was rotated after each replicate of an experiment in order to rule out any bias for one side. Individual flies were introduced into the olfactometer and observed for 5 min. First choice and total residence time in each arm was observed and analyzed using Odorifferous™ (Brundage Inc, Bryan, TX, USA). The software determines initial choice, and residence time for regions of the olfactometer. Flies were removed and killed after each test, and females were dissected to determine ovarian status. Ovarian status was assessed according to Avancini 1986 (Avancini 1986). All females that reached the latest stage of ovarian development were considered "gravid," while those that had not reached this stage were considered "non-gravid." Tests ran from 0800 to 1800 h. Flies which did not choose either arm of the olfactometer within the 5 min time span were removed from the data

set and replaced with a new fly observation.

Statistical Analysis. Data on initial choice were analyzed using a Chi Square analysis with significance observed at the $\alpha = 0.05$ level (SPSS 2010). Odifferous™ (Brundage Inc., Bryan, TX, USA) calculated the total time spent in any given region of the olfactometer, and these data were analyzed with SPSS 17 (SPSS 2010). A Wilcoxson-Signed-Rank test and a Monte Carlo simulation were used to compare the proportion of time spent in each arm of the olfactometer. Data figures were created and managed in GraphPad Prism (GraphPad Software, Inc. La Jolla, CA, USA).

Results

First choice response data yielded predominately non-significant behavior responses by *C. macellaria* (Fig. 3.2). Females with gravid exposed to fresh liver vs. no resource did not exhibit a significant first choice for either treatment ($\chi^2 = 0.50$; df = 1; $P = 0.48$). *C. macellaria* females with undeveloped ovaries had a significant first choice for the arm associated with fresh resource ($\chi^2 = 7.0$; df = 1; $P = 0.01$) while males did not exhibit a significant first choice either treatment ($\chi^2 = 0.03$; df = 1; $P = 0.86$). *C. macellaria* females with fully developed ($\chi^2 = 0.56$; df = 1; $P = 0.47$) or undeveloped ovaries ($\chi^2 = 0.00$; df = 1; $P = 1.000$), and males ($\chi^2 = 1.72$; df = 1; $P = 0.19$) did not exhibit a significant first choice to putrefied liver or the no resource treatment. Neither *C. macellaria* females with fully developed ($\chi^2 = 0.04$; df = 1; $P = 0.84$) or undeveloped ovaries ($\chi^2 = 0.00$; df = 1; $P = 1.00$), or males ($\chi^2 = 1.000$; df = 1; $P = 0.32$) had significant first responses to putrefied or fresh resources when given this choice (Fig

3.2).

Similar results were determined for first response of *Ch. rufifacies*. Females with gravid ($\chi^2 = 0.31$; df = 1; $P = 0.577$); females with undeveloped ovaries ($\chi^2 = 0.00$; df = 1; $P=1.00$); and males ($\chi^2 = 0.15$; df = 1; $P=0.70$) exposed to fresh resource vs. no resource exhibited a first choice not significantly different from random. *Chrysomya rufifacies* gravid females trended towards choosing no resource over putrefied resource, but this difference was not significant (χ^2 (1, $N = 50$) = 3.200; $P = 0.074$). Both gravid *C. rufifacies* (χ^2 (1, $N=50$) = 0.860; $P = 0.436$), and males χ^2 (1, $N = 50$) = 0.360; $P = 0.549$) exhibited no significant first choice preference for either putrefied resource or no resource. Both gravid (χ^2 (1, $N = 50$) = 3.571; $P = 0.059$) and non-gravid *Ch. rufifacies* (χ^2 (1, $N = 50$) = 2.286; $P = 0.131$) trended towards a first choice preference of putrefied resource over fresh resource yet these differences were not significant. *Ch. rufifacies* males, however, showed significant first choice preference for putrefied resource over fresh (χ^2 (1, $N = 50$) = 9.000; $P = 0.004$) (Fig. 3.2).

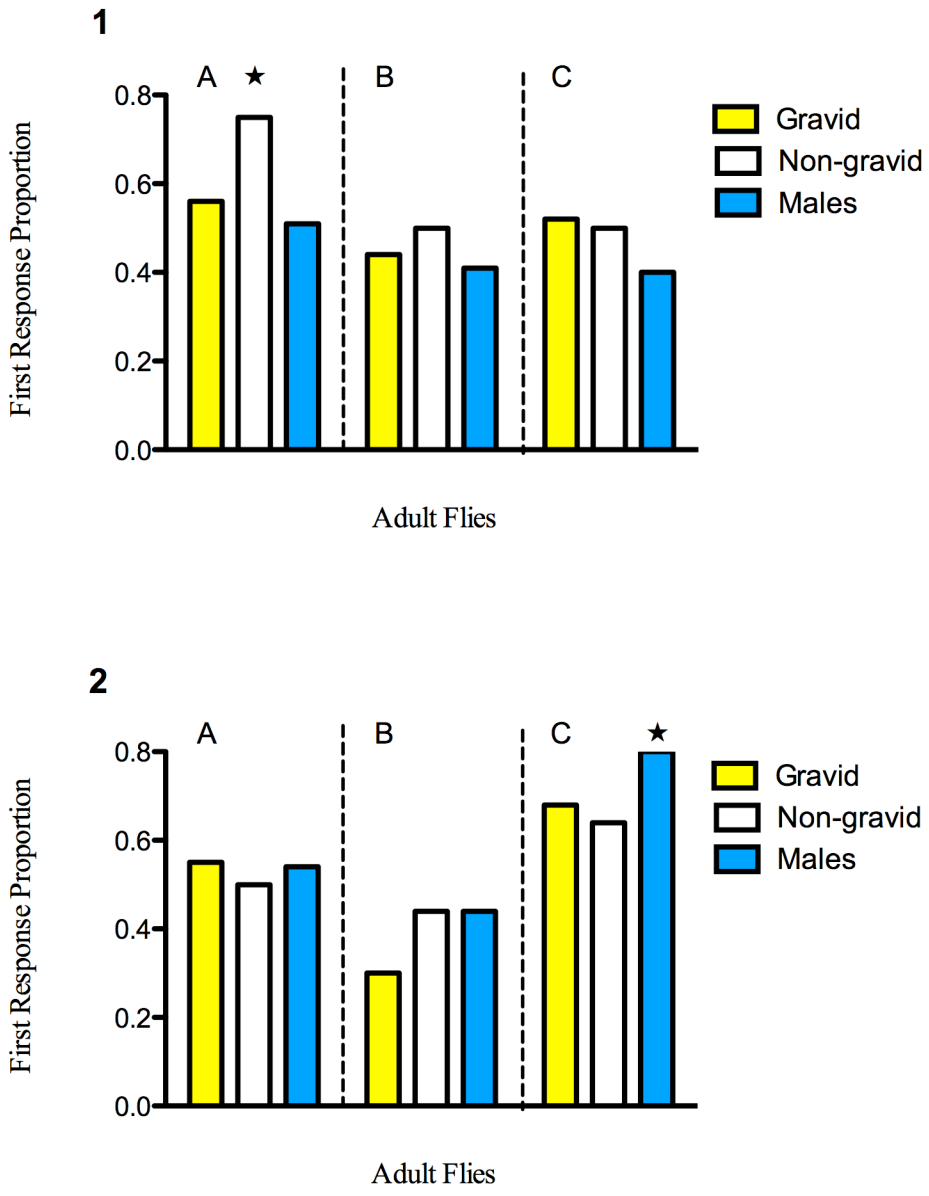


Fig 3.2. First response of proportions of 1) *Cochliomyia macellaria* and 2) *Chrysomya rufifacies* to A) fresh liver vs blank; B) putrefied liver vs blank; and C) putrefied liver vs fresh. * indicates responses significantly ($P \leq 0.05$) different from random.

Measuring residence time in the olfactometer arms corresponding to specific treatments was much more informative. Wilcoxson-Signed-Rank tests and subsequent Monte Carlo simulations indicated additional significant differences in fly response to treatments based on ovarian status (Fig. 3.3). Gravid *C. macellaria* (z = 37.61; P = 0.027) and non-gravid *C. macellaria* (z = 33.79; P = 0.015) spent significantly more time in areas associated with fresh liver over blanks, while males showed no significant difference in residence time (z = -0.992; P = 0.310). Neither gravid *C. macellaria* (z = -0.776; P = 0.434), non-gravid *C. macellaria* (z = -1.292; P = 0.099), nor males (z = -1.624; P = 0.055) exposed to putrefied resource vs. no resource showed any significant difference in residence time, although males and undeveloped females trended towards the no resource arm. Finally, neither gravid C. macellaria (z = -0.615; P = 0.537), non-gravid C. macellaria (z = -0.907; P = 0.366), nor males (z =-0.342; P = 0.740) exposed to putrefied resource vs. fresh resource spent significantly great time in either arm (Fig. 3.3).

Chrysomya rufifacies gravid females (z = -1.932; P = 0.027) spent significantly more time in areas associated with fresh resource over no resource, while non-gravid females spent significantly more time (z = -2.089; P = 0.018) in areas associated with no resource over fresh resource. *Chrysomya rufifacies* males showed no significant difference in residence time between areas associated with fresh resource and those associated with no resource (z = -0.559; P = 0.573).

Chrysomya rufifacies gravid females (z = -1.784; P = 0.034) spent significantly more time in areas associated with putrefied resource vs. no resource. Neither *Ch. rufifacies* non-gravid females (z = -0.654; P = 0.520) or males (z = -0.787; P = 0.435) spent significantly more time in either areas associated with putrefied resource or areas associated with no resource. *Chrysomya rufifacies* gravid females (z = -2.349; P = 0.018) and males (z = -1.811; P = 0.035) spent significantly more time in areas associated with putrefied resource over those associated with fresh. Non-gravid *Ch. rufifacies* (z = 33.78; P = 0.054) showed no significant difference in residence time between areas associated with fresh resource and those associated with putrefied resource (Fig. 3.3).

Discussion

First response and residence time were recorded in these experiments with the response of flies varying depending on the variable measured. First response appears to be much less informative than residence time. While significance was achieved in the first choice analyses of *C. macellaria* females and *Ch. rufifacies* males (Fig. 3.2), observed activity in the olfactometer indicated a need for acclamation to the environment before an "informed" choice could be made. First choice analysis confirms this observation, yielding nearly random first choice data in all but two experiments treatments. Most adults spent the initial 30 s of exposure in the olfactometer exploring the environment before resting in an area associated with particular bait. Therefore, all discussion will be based on residence time responses.

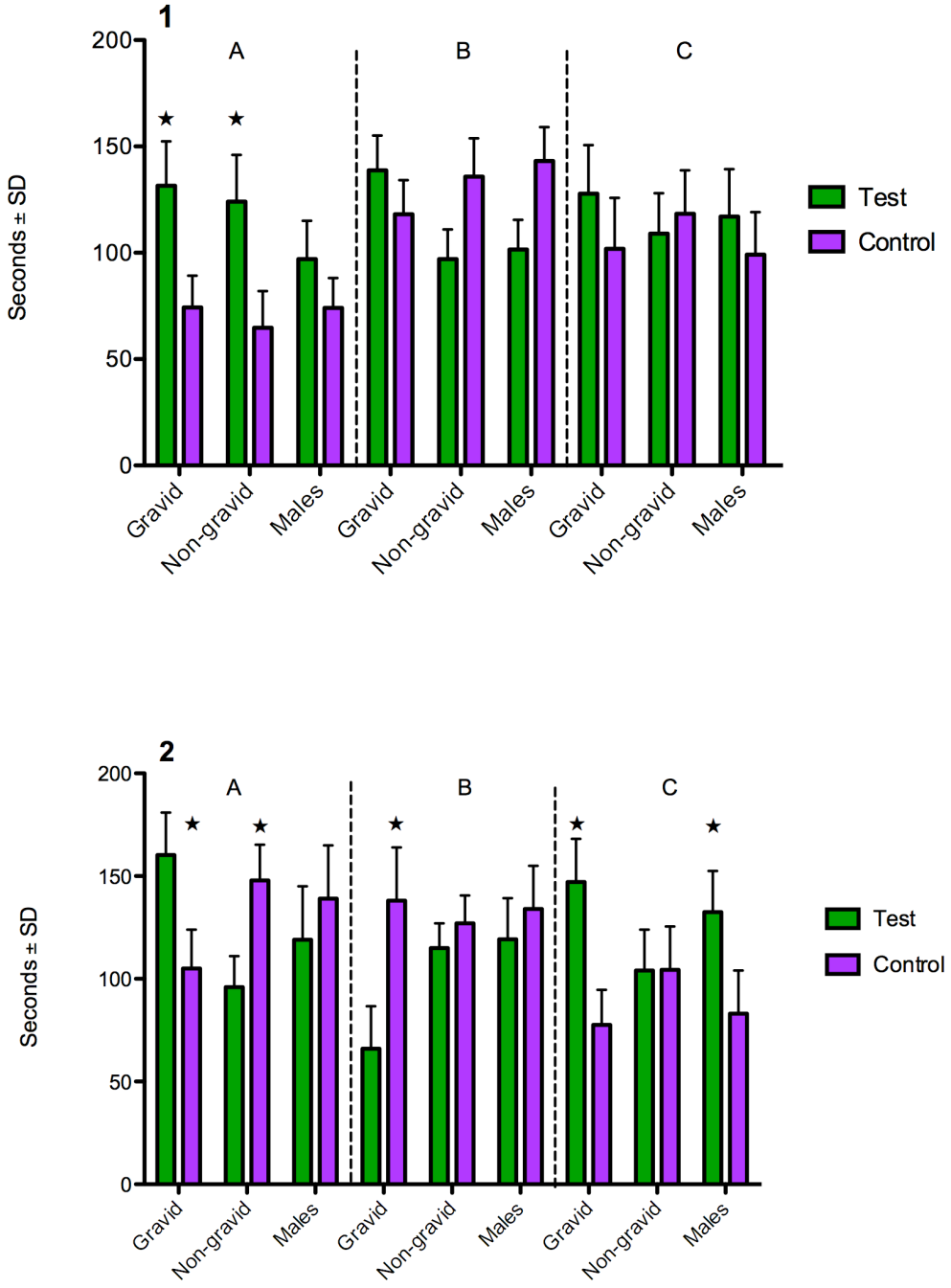


Fig 3.3. Time in seconds (mean ± SE) spent in either test or control arm for 1) *Cochliomyia macellaria* and 2) *Chrysomya rufifacies* to A) fresh liver (test) vs blank (control); B) putrefied liver (test) vs blank (control); and C) putrefied liver (test) vs fresh (control). * indicates significant ($P \leq 0.05$) difference in residence time.

As demonstrated in this study with *C. macellaria* and *Ch. rufifacies*, primary and secondary myiasis producers use differing semiochemicals to locate and colonize hosts (Aak et al. 2010). *Cochliomyia hominivorax* (Coquerel) (Diptera: Calliphoridae), the primary screw worm, is attracted to solutions of indol, skatole, and phenol, all volatiles associated with bacterial decomposition (Grabbe and Turner 1973, Hammack and Holt 1983). Similarly, *L. cuprina* and *L. sericata* females were significantly more attracted to sheep emitting volatiles such as indol and phenol than those that were not associated with such volatiles (Ashworth and Wall 1994). The results of these studies were replicated both in the lab (Grabbe and Turner 1973) and in the field (Ashworth and Wall 1994), indicating the importance of semiochemicals as a mechanism governing attraction response of adult flies.

Bacteria present on the resource most likely are the primary source of the semiochemicals utilized by blow flies to locate resources. *Cochliomyia homnivorax* adults are attracted to bacterially infected blood, and exhibited a 77% increase in landing events on blood incubated for at least 48 h. After 96 h of incubation, however, attraction dropped by 67%, indicating a change in VOCs profile as bacteria aged (Chaudhury et al. 2010). Similarly, decomposition of sheep wool by *Pseudomonas aeruginosa* results in the production of sulphurous compounds (Ashworth and Wall 1994) elicited the searching behavior by *L. sericata* adults which continued until the compounds dissipated (Wall 1994). Sulfurous compounds are also highly attractive to many other calliphorid species (Gill and Penney 1979, Ashworth and Wall 1994) including those considered primarily colonizers of carrion (Aak et al. 2010). These compounds are most

prevalent during the early to mid-stages of decay (Statheropoulos et al. 2005) and are no longer detected during the concluding stages of decay (Vass 2002, Dekeirsschieter et al. 2009).

This ability to use VOCs to locate resources could explain the successional patterns observed for Diptera colonizing carrion. For instance, carrion in Hawaii attracts *Lucilia cuprina* (Wiedemann) (Diptera: Calliphoridae) during the fresh stages of decomposition, and *Chrysomya megacephala* (Fabricius) (Diptera: Calliphoridae) and *Chrysomya rufifacies* (Macquart) (Diptera: Calliphoridae) during bloat (Early and Goff 1986) Similarly, *Chrysomya regalis* (Robineau-Desvoidy) (Diptera: Calliphoridae) arrives during the fresh stage of decomposition, while *Chrysomya abliceps* (Wiedemann) (Diptera: Calliphoridae) arrives as the carrion is entering into bloat (Braack 1987). This disparity in arrival time indicates differing cues used by each species to locate appropriate resources for colonization resulting in greater offspring survivorship (Schoenly and Reid 1987, Greenberg 1991, Anderson and VanLaerhoven 1996, Archer and Elgar 2003, Battan Horenstein et al. 2010). *C. macellaria* females, both gravid and non-gravid, gave a positive response to fresh liver when presented alone. This result is not surprising as *C. macellaria* has been shown to be a primary colonizer in the US (Hall 1993, De Jong and Hoback 2006, Gruner et al. 2007), Central America (Jiron 1981), and South America (Gomes et al. 2009, Battan Horenstein et al. 2010). One would therefore expect these flies to be attracted to resource in the early stages of decay. However, these results do not demonstrate acceptance of the resource as an oviposition site.

Cochliomyia macellaria did not demonstrate a preference for fresh or putrid liver when given a choice. Olfaction might not be an independent mechanism-regulating acceptance of a resource. Gomes et al. (2007) found that *Ch. megacephala* used vision in concert with olfaction to evaluate and accept a potential landing site. Laboratory tests showed a significant increase of 30% landing on high contrast landing sites versus patterned or low contrast landing sites (Gomes et al. 2007). Similarly, *L. sericata* attraction to high-contrast visual cues when associated with odors of liver increased by 50% over either visual or olfactory cues alone (Wall and Fisher 2001). In addition to visual stimuli, calliphorids may use tactile stimuli to evaluate a potential oviposition source. In laboratory tests, *P. regina* females oviposited 70% more often when tactilely stimulated than those who were not (Wallis 1962). This response is due to the presence of tactile sensilla on the tarsi and ovipositor (Wallis 1962). It is therefore possible that while olfaction may be an important mechanism in the acceptance of a resource for oviposition, scent may need to be compounded by additional stimuli before full acceptance is reached.

Response of *Ch. rufifacies* females to the resources examined corresponded with ovarian status. Gravid *Ch. rufifacies* were repelled when only provided putrefied liver as a choice. This is counter-intuitive, since *Ch. rufifacies* is a secondary colonizer, and shows significant levels of attraction to carrion in the later stages of decay. However, when given a choice between fresh and putrid liver, gravid *Ch. rufifacies* females spent more time in areas associated with putrid, rather than fresh, liver. Based on these results, the resource might only provide part of the stimulus necessary for females to exhibit

attraction as *Ch. rufifacies* larvae are facultative predators. (Baumgartner 1993). Therefore, *Ch. rufifacies* adults might respond to resources already infested with heterospecific larvae than resources without.

Male *C. macellaria* and *C. rufifacies* did not show significant attraction to fresh or putrid liver, which may indicate that either males do not locate resources using olfaction, or they have little need to locate a large protein resource. Male blow flies require a protein meal in order to produce sperm (Aak and Knudsen 2011) although protein requirements are significantly less than females (Stoffolano et al. 1995). Stoffolano et al. (1995) found that protein-starved *P. regina* males were able to meet dietary needs by feeding on fecal specs of protein fed females. Therefore, while protein is necessary for proper male development, it is not needed in such quantities that carrion resource location is vital for their survivorship. Males may be attracted to carrion to locate a mate, however. Gruner et al. (2007) collected males arrived at carrion, but in smaller numbers than ovipositing females (< 3% of overall trap numbers). It does not appear that decomposition state of the resource is what attracts the males to a carrion resource. Either the males are attracted to carrion regardless of decomposition state, or they use another cue.

The mechanisms that govern the attraction to an oviposition resource by calliphorid flies need to be understood completely. This knowledge will lead to a deeper understanding of colonization mechanisms, which, in turn, may be applied to such diverse applications as forensic entomology and livestock pest management. Since calliphorids are important in both industries, the knowledge of oviposition preference

could lead to a better time of colonization estimation in cases of human death, mechanisms for control of myiasis producing flies in livestock, and the development of more attractive traps for monitoring fly populations.

CHAPTER IV

ATTRACTION OF ADULT *COCHLIOMYIA MACELLARIA* AND *CHRYSOMYA RUFIFACIES* TO CONSPECIFIC AND HETEROSPECIFIC EGGS

Introduction

Blow flies (Diptera: Calliphoridae) are generally the first arthropods to colonize vertebrate carrion (Byrd and Butler 1996). Ten carrion-inhabiting blow fly species are known in Texas (McAlpine 1981, Tenorio et al. 2003). In most instances, *Calliphora vicina* (Robineau-Desvoidy) (Diptera: Calliphoridae) and *Phormia regina* (Meigen) (Diptera: Calliphoridae) colonize fresh remains during cool months (November through February)(Tenorio et al. 2003, Bucheli et al. 2009). *Cochliomyia macellaria* (Fabricius) (Diptera: Calliphoridae) and *Chrysomya rufifacies* (Macquart) (Diptera: Calliphoridae) are the dominant colonizers of carrion during warm months (Tenorio et al. 2003).

Cochliomyia macellaria and *Ch. rufifacies* larvae exploit carrion, yet temporal variation occurs with adult arrival and oviposition on carrion (Fuller 1934b, Early and Goff 1986, Eberhardt and Elliot 2008, Biavati et al. 2010). *Cochliomyia macellaria* acts as a primary colonizer, arriving within 24 h after death (Ortiz and Tomberlin 2009, Biavati et al. 2010), while *Ch. rufifacies* acts as a secondary colonizer arriving 24-48 h after death (Fuller 1934b, Early and Goff 1986, Eberhardt and Elliot 2008, Cammack et al. 2010). *Chrysomya rufifacies* larvae are also facultatively predaceous (Fuller 1934b) on *C. macellaria* larvae (Baumgartner 1993). Arrival time significantly affects the fitness of both species, as *Ch. rufifacies* are more fit if they arrive after *C. macellaria*,

while *C. macellaria* are more fit if they arrive before *Ch. rufifacies* (Brundage unpub).

Variation in time of arrival and colonization indicates cues associated with carrion are used by *C. macellaria* and *Ch. rufifacies* to locate and assess resources. These cues may be visual (Wallis 1962, Easton and Feir 1991, Collins 1996, Gomes et al. 2007), auditory (Wertheim et al. 2005, Wicker-Thomas 2007), tactile (Eismann and Rice 1987, Easton and Feir 1991), or olfactory (Crombie 1944, Browne 1960, Gomes et al. 2007), and may emanate from the resource itself (Wall and Fisher 2001, Gomes et al. 2007, Ortiz and Tomberlin 2009), or interspecifics or conspecifics inhabiting the resource (Judd 1992, Davies 1998, Diaz-Fleischer and Aluja 2003). Volatiles associated with ovipositing conspecific (Browne 1960) and interspecific females (Rosati and Laerhoven 2010) are also used to locate resources. Lam et al. (2007) determined ovipositing Muscidae (Diptera) use conspecific egg-associated volatiles to assess oviposition sites. Muscids ovipositing in conjunction with conspecifics suffered lower levels of predation than those arriving later (Lam et al. 2007). Similarly, *Lucilia cuprina* (Meigen) (Diptera: Calliphoridae) preferentially oviposit in the presence of other actively ovipositing females (Browne et al. 1968). This behavior results in maggot masses which may accelerate digestion of the resource and enhance larval feeding (Browne 1958).

Volatiles from bacteria associated with blow fly larvae are also used by conspecific and intraspecific adults to assess suitability of a resource for oviposition(Ashworth and Wall 1994). *Lucilia sericata* (Meignen) (Diptera: Calliphoridae) are attracted to *Pseudomonas aeruginosa* derived volatiles when

searching for oviposition sites. Similarly, calcium sulphide, calcium carbonate, and sodium sulphide all attract gravid *L. sericata* and *L. cuprina,* and are associated with larval bacteria (Ashworth and Wall 1994). I hypothesize *C. macellaria* and *C. rufifacies* respond to olfactory cues from conspecific and interspecific eggs and that microbes associated with the eggs serve as the primary mechanism.

Materials and Methods

Adult Fly Colony. Laboratory colonies of *C. macellaria* and *Ch. rufifacies* were initiated from specimens collected in Brazos County, Texas, USA during spring and summer of 2009 and 2010. Larvae were reared at a standard density of 100 larvae on 50 g of fresh bovine liver in 3-L plastic containers. All rearing was done in a Rheem Environmental in walk-in growth chamber (Ashville, NC, USA) at 27°C ± 1°C, 60% RH, and a 12:12 (L:D) photoperiod. Dispersing third instar larvae were transferred to 3-L containers with autoclaved sand (Town & Country Landscape Supply Co., Chicago, IL, USA) for pupation. Resulting adults were maintained in 300 cm^3 cages (Bioquip Products, Rancho Dominguez, CA, USA) held in the growth chamber previously described. Granulated sugar (Imperial Sugar Co., Sugar Land, TX, USA), buttermilk powder (Saco foods Inc., Middleton, WI, USA), and water were provided ad libitum, and 20 g bovine liver was placed in the cage between 2 and 5 d post emergence for 8 h to induce ovarian development.

Behavioral Bioassay. A dual choice olfactometer was used to evaluate the choice of calliphorid adults to fresh or aged liver. The olfactometer was comprised of three

stacked 2.7 cm thick sheets of solid Teflon®, covered by removable sheet glass (Fig. 3.1). The intake port on each arm measured 1.3 cm in diameter and was located at the terminal end. The exhaust port located at the terminal end of the stem measured 1.7 cm in diameter. A 1.6 cm access port for introducing the flies to the olfactometer was located in the base of the olfactometer approximately 2.5 cm from the terminal end. Air was pulled through the olfactometer with a 50 mm USB powered computer cooling fan (5VDC Fan, Dc Fans, Thermal Management NMB Technologies Corporation, Chatsworth, CA, USA) mounted to the external surface of the outflow port at the terminal end of the stem. Airflow through the olfactometer at the access port was measured using an anemometer (Testo 435-1, Testo, Inc., Sparta, NJ, USA) to be 0.5 m/sec (based on a 90 s average). Preliminary runs sans resource were conducted with colony flies to ensure adequate wind speed and assure lighting, temperature, and ambient odors did not induce behavioral responses. These "olfactometer integrity checks" were replicated throughout the experiment.

Air flowed through 15 mm diameter, 14.5 cm long glass tubes containing activated charcoal (Aqua-Tech, Marineland Aquarium Products, Moorpark, CA, USA) and plugged with polyester filter floss (Aqua-Tech, Marineland Aquarium Products, Moorpark, CA, USA). The columns were attached to 15 cm x 15 cm x 12 cm plastic chambers (S.C. Johnson & Son, Inc., Racine, WI, USA), which were used to hold the treatments for all experiments. Chambers were attached to olfactometer arms with 7 cm Teflon® tubing, and chambers were replaced after the completion of each experiment. Two fluorescent light tubes (60 W) served for overhead illumination. Temperature in the

olfactometer room was 24.0 ± 2.0°C. For all experiments, 50 males, 50 gravid females, and 50 non-gravid females were individually observed for five minutes to determine response to heterospecific and conspecific eggs of various ages. Twelve comparative experiments were performed for *C. macellaria* and *Ch. rufifacies* to conspecific and heterospecific eggs (Table 4.1).

Table 4.1. Behavioral bioassay experiments. Each experiment recorded the initial response and the resident time of 50 males, 50 gravid females, and 50 non-gravid females for 5 min (experiments 1-12), or the initial response and resident time of 50 gravid and 50 non-gravid females for 2 min (experiments 13-28). Experiments 1-12 represent the initial behavioral assay experiments. Experiments 13-20 represent the surface sterilized egg behavioral assay experiments. Experiments 21-28 represent the surface microbe behavioral assay experiments.

Experiment #	**Egg age**	**Species of Egg**	**Species of Adults**
1	< 3 h	*C. macellaria*	*C. macellaria*
2	< 3 h	*Ch. rufifacies*	*C. macellaria*
3	< 3 h	*C. macellaria*	*Ch. rufifacies*
4	< 3 h	*Ch. rufifacies*	*Ch. rufifacies*
5	3-6 h	*C. macellaria*	*C. macellaria*
6	3-6 h	*Ch. rufifacies*	*C. macellaria*
7	3-6 h	*C. macellaria*	*Ch. rufifacies*
8	3-6 h	*Ch. rufifacies*	*Ch. rufifacies*
9	6-9 h	*C. macellaria*	*C. macellaria*
10	6-9 h	*Ch. rufifacies*	*C. macellaria*
11	6-9 h	*C. macellaria*	*Ch. rufifacies*
12	6-9 h	*Ch. rufifacies*	*Ch. rufifacies*
13	Surface sterilized <3 h	*C. macellaria*	*C. macellaria*
14	Surface sterilized <3 h	*Ch. rufifacies*	*C. macellaria*
15	Surface sterilized <3 h	*C. macellaria*	*Ch. rufifacies*
16	Surface sterilized <3 h	*Ch. rufifacies*	*Ch. rufifacies*
17	Surface sterilized 3-6 h	*C. macellaria*	*C. macellaria*
18	Surface sterilized 3-6 h	*Ch. rufifacies*	*C. macellaria*

Table 4.1, continued

Experiment #	Egg age	Species of Egg	Species of Adults
19	Surface sterilized 3-6 h	*C. macellaria*	*Ch. rufifacies*
20	Surface sterilized 3-6 h	*Ch. rufifacies*	*Ch. rufifacies*
21	Microbes from eggs < 3 h	*C. macellaria*	*C. macellaria*
22	Microbes from eggs < 3 h	*Ch. rufifacies*	*C. macellaria*
23	Microbes from eggs < 3 h	*C. macellaria*	*Ch. rufifacies*
24	Microbes from eggs < 3 h	*Ch. rufifacies*	*Ch. rufifacies*
25	Microbes from eggs 3-6 h	*C. macellaria*	*C. macellaria*
26	Microbes from eggs 3-6 h	*Ch. rufifacies*	*C. macellaria*
27	Microbes from eggs 3-6 h	*C. macellaria*	*Ch. rufifacies*
28	Microbes from eggs 3-6 h	*Ch. rufifacies*	*Ch. rufifacies*

General Experimental Design. For both species, approximately 7-d-old flies were collected from colonies and placed individually in clean glass vials (2-dram, 40 mm height x 17 mm diameter) for sexing prior to experiments. The olfactometer was cleaned with 80% ethanol prior to the introduction of a specimen. Treatment location the olfactometer arms was rotated after each fly tested in order to rule out any bias for one side. Individual flies were introduced into the olfactometer and observed for two minutes. First choice and total residence time in each arm was observed and analyzed using Odorifferous$^{(TM)}$ (Brundage Inc, Bryan, TX, USA). The software determines initial choice, final choice and residence time for regions of the olfactometer. Flies were removed and killed after each test. Females were dissected and ovarian status recorded

following Avancini 1986 (Avancini 1986). Females with ovarioles that had not reached the maximum size were considered "non-gravid," while those that had reached the maximum size were considered "gravid" and physiologically ready to oviposit. Experiments were conducted from 0800 to 1800 h.

Egg Collection. Approximately 5000 eggs (0.5 g) on 1.0 g fresh bovine liver were used as the treatment for these experiments, while fresh liver alone served as the control. Pilot studies using the dual choice olfactometer confirmed the volatile capability of this egg mass. Eggs were collected from colonies by presenting flies with fresh bovine liver for one h. Using fresh liver as an oviposition resource allowed for the rapid collection of similarly aged eggs. Once eggs were collected, they were aged in a Rheem Environmental walk-in growth chamber 27°C.

Response to Eggs of Various Ages. Behavioral response of *C. macellaria* and *Ch. rufifacies* to conspecific and heterospecific eggs of different ages was examined. Adult flies previously described were examined for their response to eggs aged: a) < 3 h; b) 3-6 h; c) 6-9 h. The oldest time treatment was selected as it was closest to egg hatch (Byrd and Butler 1996, 1997).

Response to Non-sterile and Sterile Eggs. Eggs < 3 h and 3-6 h old resulted in significant ($P < 0.05$) behavioral responses of adult blow flies in the previous experiment. Therefore, these age groups were used to determine if attraction was governed by egg respiration or associated microbes. Results from initial egg age experiments also indicated that 2-minute observations were sufficient to determine residence time in behavioral assays. Eggs of appropriate ages were surface sterilized and

presented to individual gravid and non-gravid females in the olfactometer. Eggs were surface sterilized after Brundage et al. (unpublished data). Sterilization treatments on both *C. macellaria* and *Ch. rufifacies* eggs indicated a 10-minute Lysol® soak yielded 100% surface sterilization based on agar-based culture methods with 85% egg eclosion (Brundage, unpublished data).

Eggs were placed on a sterile Millipore 20 μm membrane filter (Millipore, Billerica, MA), and placed in a 25 mm stainless steel leur-lock filter holder (Millipore, Billerica, MA). The filter holder was attached to a sterile glass leur-lock syringe (Thermo Fisher Scientific, Waltham, MA), loaded with 20 ml Professional Lysol® Antibacterial All Purpose Cleaner Concentrate (undiluted) (Reckitt Benckiser. Parsippany, NJ). Ten ml Lysol® was washed through the filter, thereby submerging the eggs in disinfectant. Eggs were soaked for 10 min, and then rinsed in 20 ml sterile insect saline to remove residual Lysol®. Surface sterilized eggs were transferred to sterilized 15 cm x 15 cm x 12 cm plastic chambers (S.C. Johnson & Son, Inc., Racine, WI, USA) (Table 4.1).

Response to Surface Microbes. To determine if microbes and associated oviposition excretions were responsible for the behavioral response of the flies, eggs from the previous age categories identified were killed using a tissue homogenizer (Kinematica AG, Nurnberg, Germany).

In order to increase focus on microbes only associated with eggs, a sterile liver and agar mixture, adapted from Sherman 1995 (Sherman and Tran 1995) was used to collect eggs from colonies. Approximately 20 g fresh bovine liver was placed at 37°C in a

Rheem Environmental Chamber for 24 h to induce putrefaction. The putrefied liver was pureed and mixed with 20 g nutrient agar. The mixture was autoclaved and 30 ml aliquots were partitioned into sterile plastic cups (Bio-Serv, Frenchtown, NJ). Oviposition "troughs," 5 mm long, 2 mm wide, and 5 mm deep were cut into the solidified mixture to provide arms for oviposition. Cups were presented to laboratory colonies for one h to collect the requisite 5000 eggs.

Eggs were aseptically removed and placed in sterile 25 ml centrifuge tubes (Thermo Fisher Scientific, Waltham, MA). Eggs were mixed with 9 ml sterile PBS and homogenized using a PolyTron handheld tissue homogenizer (Kinematica AG, Nurnberg, Germany). The resulting liquid was filtered through a sterile, low protein binding Millipore 0.22 μm membrane filter (Millipore, Billerica, MA) to collect any microbes and other substances associated with the eggs. The filter with collected microbes was transferred to a sterile plastic chamber as described above and used for behavioral assays as previously described.

Quantification of Bacterial Diversity on Eggs Over Time. Results from the previously described experiments indicated microbes may serve as a mechanism governing blow fly attraction. In order to assess the microbial community associated with these eggs overtime, bacterial DNA was isolated from eggs representing each age group (< 3 h, 3-6 h, and 6-9 h old) and associated bacteria was identified via 454 pyrosequencing.

DNA Isolation. DNA from surface microbes was extracted from egg samples of each species and each egg age using a modified phenol extraction method. Eggs were aseptically collected as described above, and 0.01 g eggs of each age from each species

was placed in individual Fisherbrand microcentrifuge tubes (Thermo Fisher Scientific, Waltham, MA) and stored at -80°C. Eggs were thawed and crushed using Fisherbrand Pellet Pestles (Thermo Fisher Scientific, Waltham, MA). Crushed tissues were suspended in 567 ml TE buffer. 30 µl 10% SDS was added to the mixture, with 3 µl 20mg/ml proteinase K, and 10 µl lysozyme. The mixture was incubated at 37 C for one h. 100 µl 5M NaCl was added, along with 80 µl CTAB/NaCL solution. Mixture was incubated for 10 min at 65°C. Equal volumes of chloroform and isoamyl alcohol were added, and the mixture centrifuged at 24,500 x g for 5 min. Supernatant was decanted into a fresh tube mixed with an equal volume of phenol, chloroform, and isoamyl alcohol. The mixture was centrifuged at 24,500 x g for 5 min. Supernatant was decanted into a fresh tube and 0.6 ml isoproponol was added. The tube was manually shaken to precipitate the nucleic acids, and then centrifuged at 4000 x g for 2 minutes. The pellet was transferred to a clean tube.

Pyrosequencing. DNA products were subject to 454 pyrosequencing performed by Research and Testing Laboratory (Lubbock, TX), and analyzed on the Genome Sequencer FLX instrument using Titanium protocols and reagents (Roche, Indianapolis, IN) as described by Dowd et al. (Dowd et al. 2008).

Sequences were identified to their closest operational taxonomic units (OTUs) at a minimum of 80% identity using a naïve Bayesian classifier, the Ribosomal Database Project (RDP) curated by the Center for Microbial Ecology at Michigan State University (Wang et al. 2007). All sequences that did not match at the minimum of 80% identity were discarded and were assumed to be of poor quality or derived from unclassified

bacteria.

Statistical Analysis. Data on initial choice were analyzed using a Chi Square analysis with significance observed at the $\alpha = 0.05$ level. Odorifferous$^{(TM)}$ provided some basic calculations such as the total time spent in any given region of the olfactometer, and these data were inputted into SPSS 17 (SPSS 2010) for further analysis. A Wilcoxson-Signed-Rank test and a Monte Carlo simulation were used to compare the proportion of time spent in each arm of the olfactometer. Data figures were created and managed in GraphPad Prism (GraphPad Software, Inc. La Jolla, CA, USA).

Results

Egg Age Behavioral Bioassay: Conspecific Response to Eggs < 3 h Old. Data for fly responses to fresh eggs (< 3 h old) yielded predominantly non-significant first choice response, with a few notable exceptions. *Chrysomya rufifacies* gravid females exposed to conspecific eggs were significantly attracted to the eggs over the control ($\chi^2 = 5.488$; df = 1; $P = 0.019$). Neither *Ch. rufifacies* non-gravid females ($\chi^2 = 0.235$; df = 1; $P = 0.628$) nor males ($\chi^2 = 1.280$; df = 1; $P = 0.258$) were significantly attracted to either conspecific eggs or the control according to first choice data. Based on first choice, gravid *C. macellaria* did not significantly respond to conspecific fresh eggs ($\chi^2 = 0.067$; df = 1; $P = 0.796$), nor did non-gravid females ($\chi^2 = 1.373$; df = 1; $P = 0.241$) or males ($\chi^2 = 0.831$; df = 1; $P = 0.362$) (Fig. 4.1).

Wilcoxon-rank-sum tests and subsequent Monte Carlo simulations of residence time indicated additional significant differences in fly response to treatments based on

sex and ovarian status. Statistical tests also revealed some differences between first choice and residence time. *Chrysomya rufifacies* gravid females exposed to conspecific eggs spent significantly more time in olfactometer arms associated with eggs over the control (z = -2.126; P = 0.016). Neither *Ch. rufifacies* non-gravid (z = -0.369; P = 0.352) nor males (z = -0.014; P = 0.493) were significantly attracted to either conspecific eggs or the control. *Cochliomyia macellaria* adults presented with conspecific fresh eggs did not show significant residence time response for gravid females (z = -0.897; P = 0.187) or males (z = -0.521; P = 0.299), but non-gravid females (z = -1.714; P = 0.044) spent significantly more time in arms associated with conspecific eggs (Fig. 4.1).

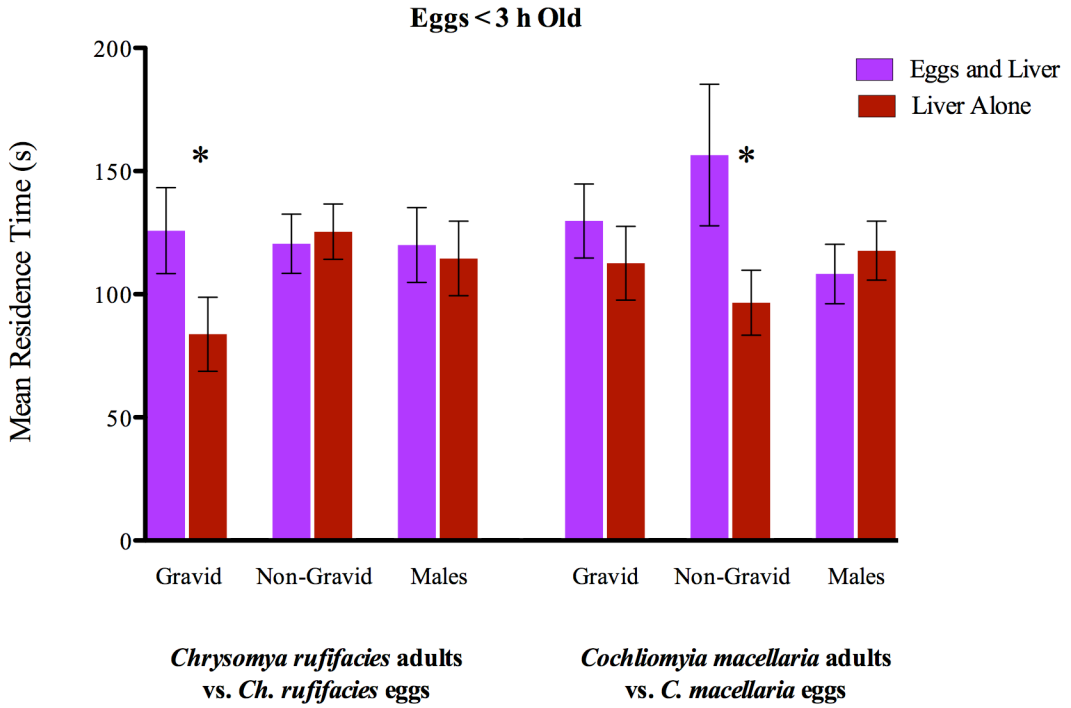


Fig 4.1. Mean residence time (mean ± SE) of adult flies presented with conspecific eggs < 3 h old in the dual choice olfactometer. * indicates significant ($P \leq 0.05$) difference in residence time.

Heterospecific Response to Eggs < 3 h Old. *Chrysomya rufifacies* adults presented with fresh *C. macellaria* eggs exhibited some significant first choice data. First choice results for gravid (χ^2 = 3.125; df = 1; P = 0.077) and pre-gravid females (χ^2 = 0.074; df = 1; P = 0.785) were not significant, but response for males was significant for fresh heterospecific eggs (χ^2 = 5.255; df = 1; P = 0.022). *Cochliomyia macellaria* adults presented with heterospecific eggs showed no significant first choice data gravid females (χ^2 = 0.510; df = 1; P = 0.475), non-gravid females (χ^2 = 0.065; df = 1; P = 0.799), or males (χ^2 = 0.020; df = 1; P = 0.889) (Fig. 4.2).

Chrysomya rufifacies adults presented with fresh *C. macellaria* eggs exhibited significant residence time responses. Gravid females (z = -2.368, P = 0.009) spent significantly more time in arms associated with heterospecific eggs, while non-gravid females (z = -1.337, P = 0.089) and males (z = -1.531, P = 0.062) showed no significant difference in residence time. *Cochliomyia macellaria* gravid presented with heterospecific eggs spent significantly more time in arms associated with controls over eggs (z = -3.838, P < 0.0001), while non-gravid females (z = -0.377, P = 0.362) and males (z = -1.047, P = 0.144) showed no significant difference in residence time (Fig. 4.2).

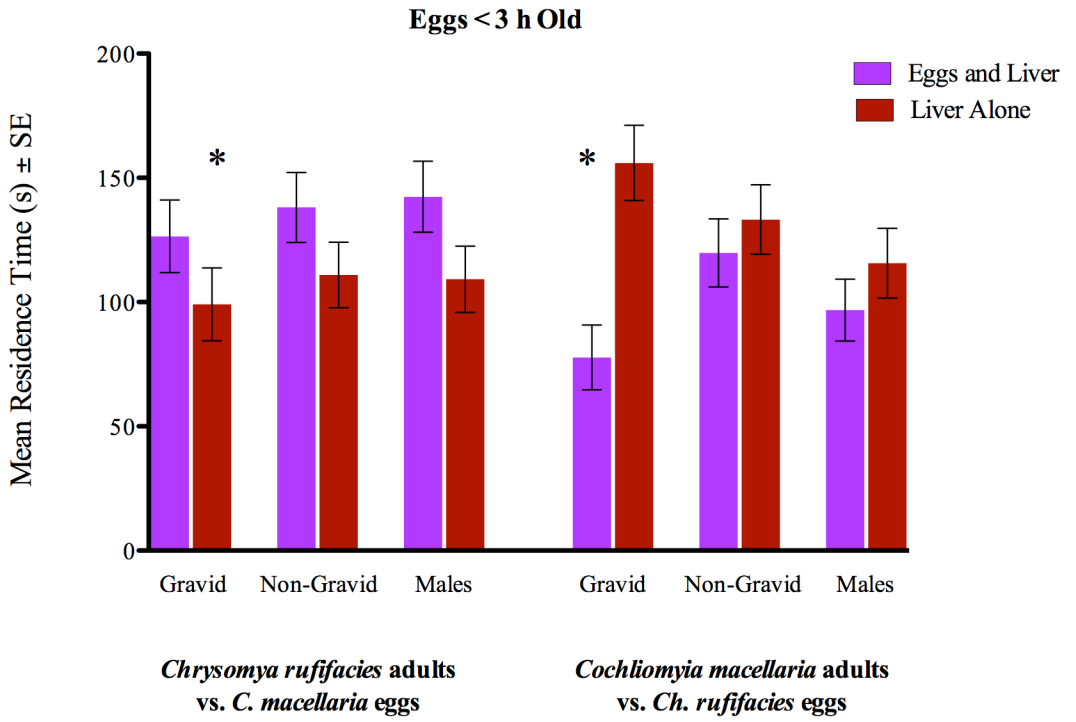


Fig 4.2. Mean residence time (mean ± SE) of adult flies presented with heterospecific eggs < 3 h old in the dual choice olfactometer. * indicates significant ($P \leq 0.05$) difference in residence time.

Conspecific Response to Eggs 3-6 h Old. Adult response to conspecific eggs aged 3-6 h yielded insignificant first choice response data. *Chrysomya rufifacies* adults exposed to conspecific eggs showed no significant attraction to the eggs or control for gravid females ($\chi^2 = 0.074$; df = 1; $P = 0.785$), non-gravid females ($\chi^2 = 0.914$; df = 1; $P = 0.339$) or males ($\chi^2 = 2.000$; df = 1; $P = 0.157$). *Cochliomyia macellaria* adults presented with conspecific eggs also did not show significant first choice response for gravid females ($\chi^2 = 3.169$; df = 1; $P = 0.075$), non-gravid females ($\chi^2 = 0.111$; df = 1; $P = 0.739$), or males ($\chi^2 = 0.000$; df = 1; $P = 1.000$) (Fig. 4.3).

Wilcoxon-rank-sum tests and subsequent Monte Carlo simulations analyzing residence time data indicated one significant difference in fly response to treatments

based on sex and ovarian status. Gravid *Ch. rufifacies* females (z = -0.211; P = 0.417), non-gravid females (z = -0.195; P = 0.428) and males (z = -1.374; P = 0.084) did not spend significantly more time in olfactometer arms associated with conspecific eggs over those associated with the control. Gravid *C. macellaria* adults (z = -2.965; P = 0.002) spent significantly more time in arms associated conspecific eggs than the control, while non-gravid females (z = -0.395; P = 0.348) and males showed no significant residence time response (z = -0.334; P = 0.367) (Fig. 4.3).

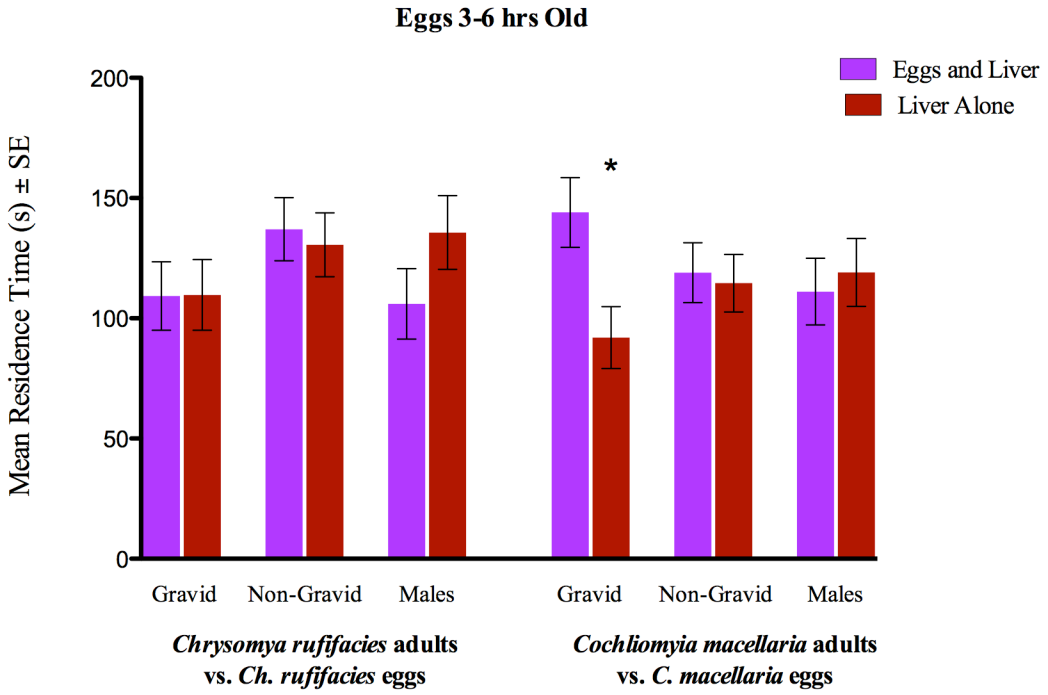


Fig 4.3. Mean residence time (mean ± SE) of adult flies presented with conspecific eggs 3-6 h old in the dual choice olfactometer. * indicates significant ($P \leq 0.05$) difference in residence time.

Heterospecific Response to Eggs 3-6 h Old. *Chrysomya rufifacies* adults presented with *C. macellaria* eggs exhibited no significant first choice data for gravid females (χ^2 = 0.720; df = 1; P = 0.396), non-gravid females (χ^2 = 1.250; df = 1; P = 0.264), or males

(χ^2 = 0.860; df = 1; P = 0.354). Gravid *C. macellaria* females presented with heterospecific eggs were significantly attracted to the control (χ^2 = 6.480; df = 1; P = 0.011), while non-gravid females (χ^2 = 0.267; df = 1; P = 0.703) and males (χ^2 = 0.080; df = 1; P = 0.777) showed no significant first choice (Fig. 4.4).

Residence time analysis yielded gravid *Ch. rufifacies* spent significantly more time in arms associated with *C. macellaria* eggs (z = -1.628; P = 0.049). Non-gravid females (z = -0.789; P = 0.209) and males (z = -1.282; P = 0.099), however, both show no significant difference in residence time. *Cochliomyia macellaria* adults presented with heterospecific eggs showed no significant residence time data for gravid females (z = -1.374, P = 0.084), non-gravid females (z = -1.192; P = 0.122), or males (z = -1.041; P = 0.148) (Fig. 4.4).

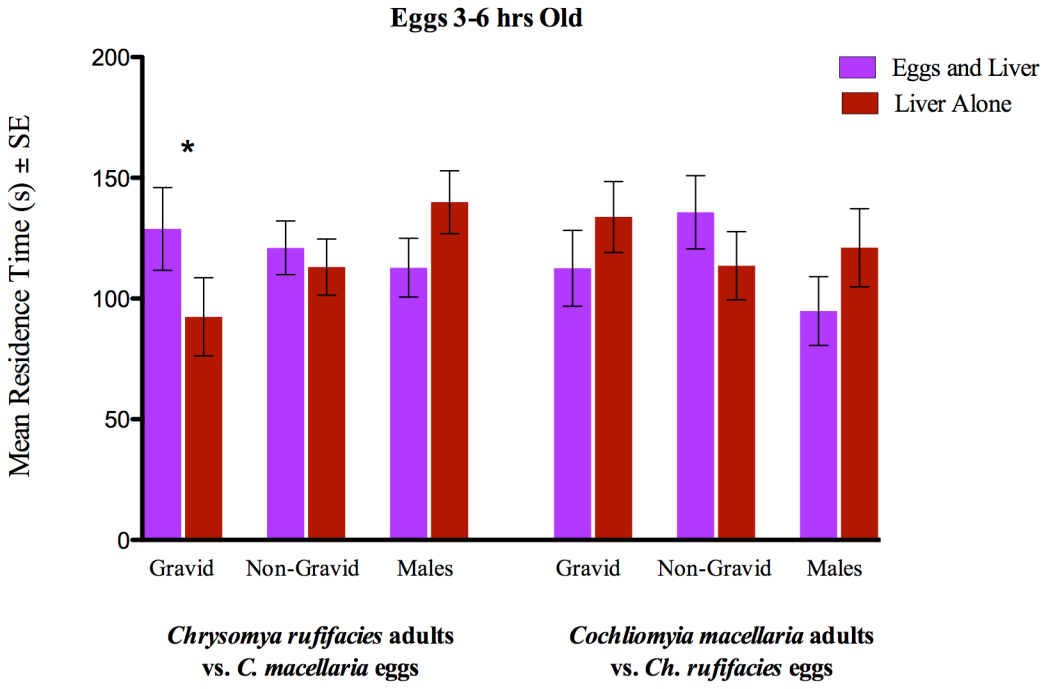


Fig 4.4. Mean residence time (mean ± SE) of adult flies presented with heterospecific eggs 3-6 h old in the dual choice olfactometer. * indicates significant ($P \leq 0.05$) difference in residence time.

Conspecific Response to Eggs 6-9 h Old. Eggs aged 6-9 h yielded all insignificant first choice responses. *Chrysomya rufifacies* adults exposed to conspecific eggs showed no significant attraction to the eggs and liver treatment over the liver alone for females with gravid females ($\chi^2 = 0.080$; df = 1; $P = 0.777$), non-gravid females ($\chi^2 = 0.220$; df = 1; $P = 0.759$) or males ($\chi^2 = 0.818$; df = 1; $P = 0.462$). *Cochliomyia macellaria* adults presented with conspecific eggs also did not show significant first choice response for fully developed adults ($\chi^2 = 0.926$; df = 1; $P = 0.447$), non-gravid ($\chi^2 = 0.563$; df = 1; $P = 0.544$), or males ($\chi^2 = 0.806$; df = 1; $P = 0.369$) (Fig. 4.5).

Wilcoxon-rank-sum tests and subsequent Monte Carlo simulations analyzing residence time data also did not indicate significant differences in fly response to treatments based on sex or ovarian status. Gravid *Ch. rufifacies* adults ($z = -0.064$; $P = 0.472$) and males ($z = -0.160$; $P = 0.439$) did not spend significantly more time in olfactometer arms associated with conspecific eggs over those associated with liver alone, while females with non-gravid ovaries ($z = -1.694$; $P = 0.041$) spent more time in arms associated with conspecific eggs. Gravid *C. macellaria* females ($z = -2.075$; $P = 0.051$), non-gravid females ($z = -1.89$; $P = 0.25$) and males ($z = -1.14$; $P = 0.23$) showed no significant residence time response (Fig. 4.5).

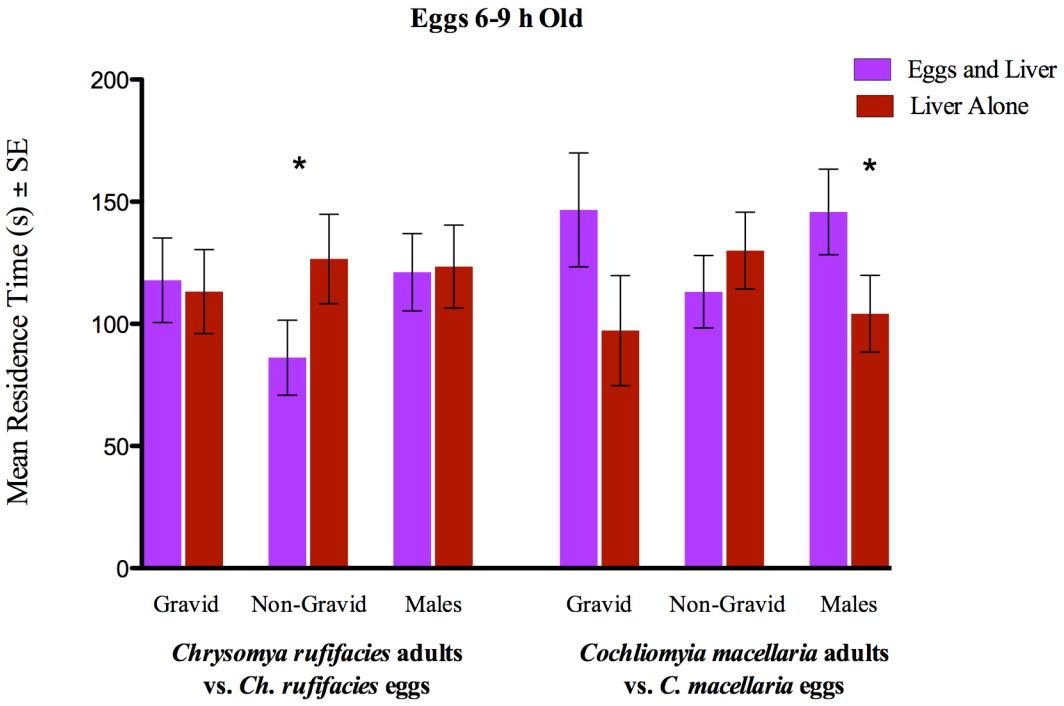


Fig 4.5. Mean residence time (mean ± SE) of adult flies presented with conspecific eggs 6-9 h old in the dual choice olfactometer. * indicates significant ($P \leq 0.05$) difference in residence time.

Heterospecific Response to Eggs 6-9 h Old. *Chrysomya rufifacies* adults presented with *C. macellaria* eggs exhibited no significant first choice data for fully developed females ($\chi^2 = 1.29$; df = 1; $P = 0.26$), non-gravid females ($\chi^2 = 0.00$; df = 1; $P = 1.00$), or males ($\chi^2 = 0.00$; df = 1; $P = 1.00$). *Cochliomyia macellaria* adults presented with heterospecific eggs showed no significant first choice for developed females ($\chi^2 = 0.93$; df = 1; $P = 0.37$), non-gravid females ($\chi^2 = 0.36$; df = 1; $P = 0.55$) or males ($\chi^2 = 0.08$; $P = 0.78$) (Fig. 4.6).

Residence time data showed that *Ch. rufifacies* fully developed females ($z = -1.81$; $P = 0.07$), non-gravid females ($z = -0.253$; $P = 0.393$), and males ($z = -0.93$; $P = 0.18$) showed no significant difference in residence time when presented with

heterospecific eggs. *Cochliomyia macellaria* adults presented with heterospecific eggs showed no significant residence time data for fully developed females (z = -0.86; P = 0.19), non-gravid females (z = -0.08; P = 0.47), or males (z = -0.18; P = 0.44) (Fig. 4.6).

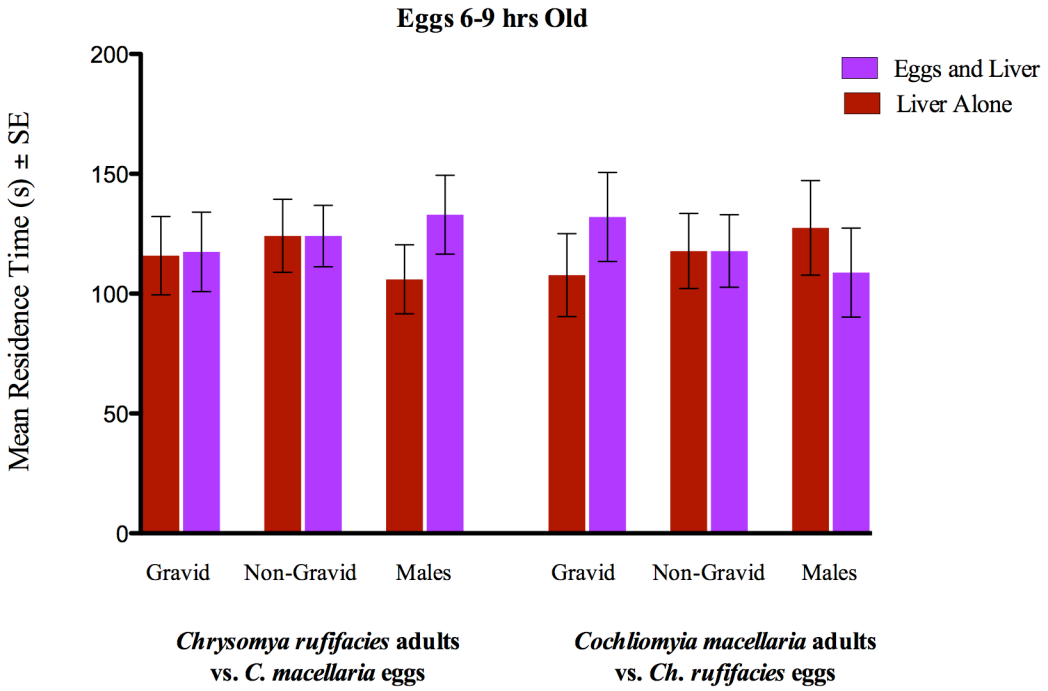


Fig 4.6. Mean residence time (mean ± SE) of adult flies presented with heterospecific eggs 6-9 h old in the dual choice olfactometer. * indicates significant ($P \leq 0.05$) difference in residence time.

Conspecific Response to Surface-Sterilized Eggs < 3 h Old. Sterile fresh eggs (< 3 h old) yielded predominantly insignificant first choice response data, with one exception. Gravid *Ch. rufifacies* exposed to conspecific surface-sterilized eggs were not significantly attracted to the eggs over the control (χ^2 = 2.57; df = 1; P = 0.11). Non-gravid *Ch. rufifacies*, however, were significantly attracted to conspecific sterile eggs (χ^2 = 5.16; df = 1; P = 0.02) according to first choice data. *Cochliomyia macellaria* adults

presented with conspecific surface-sterilized fresh eggs did not show significant first choice response for either fully developed females ($\chi^2 = 0.50$; df = 1; $P = 0.48$) or non-gravid females ($\chi^2 = 3.60$; df = 1; $P = 0.06$) (Fig. 4.7).

Wilcoxon-rank-sum tests and subsequent Monte Carlo simulations analyzing residence time data indicated significant differences in fly response to treatments based on ovarian status. Gravid *Ch. rufifacies* ($z = -1.63$; $P = 0.05$) and non-gravid ($z = -2.82$; $P = 0.002$) exposed to conspecific surface-sterilized eggs spent significantly more time in olfactometer arms associated with eggs. *Cochliomyia macellaria* adults presented with conspecific surface-sterilized fresh eggs did not show significant residence time response for fully developed adults ($z = -0.76$; $P = 0.22$), but non-gravid females ($z = -2.27$; $P = 0.01$) spent significantly more time in arms associated with the control over those associated with eggs (Fig. 4.7).

Heterospecific Response to Surface-Sterilized Eggs < 3 h Old. *Chrysomya rufifacies* adults presented with surface-sterilized fresh *C. macellaria* eggs did not show significant first choice response for either fully developed females ($\chi^2 = 3.27$; df = 1; $P = 0.07$) or pre-gravid females ($\chi^2 = 2.46$; df = 1; $P = 0.12$). *Cochliomyia macellaria* adults presented with heterospecific eggs showed no significant first choice data for fully developed females ($\chi^2 = 0.00$; df = 1; $P = 1.00$) or non-gravid females ($\chi^2 = 0.68$; df = 1; $P = 0.41$) (Fig. 4.8).

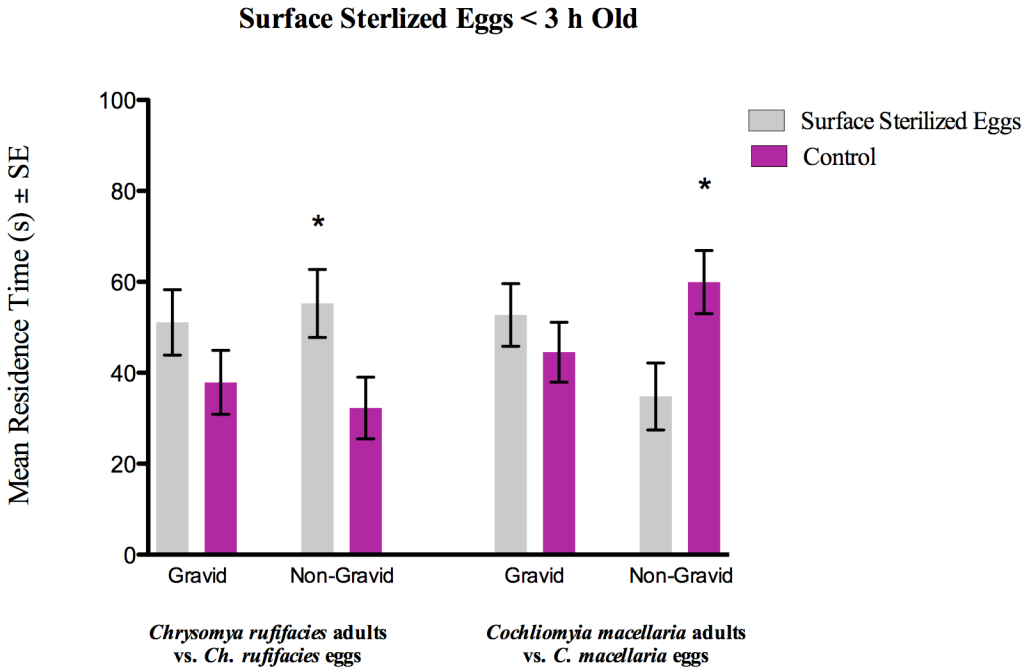


Fig 4.7. Mean residence time (mean ± SE) of adult flies presented with surface-steralized conspecific eggs < 3 h old in the dual choice olfactometer. * indicates significant ($P \leq 0.05$) difference in residence time.

Chrysomya rufifacies fully developed (z = -3.93; $P < 0.0001$) and non-gravid females (z = -1.76; $P = 0.040$) presented with surface-sterilized fresh *C. macellaria* eggs both spent significantly more time in arms associated with eggs over those associated with the control. Gravid *C. macellaria* presented with surface-sterilized heterospecific eggs spent significantly more time in arms associated with eggs (z = -2.26; $P = 0.013$) while non-gravid females showed no significant difference in residence time (z = -1.24; $P = 0.10$) (Fig. 4.8).

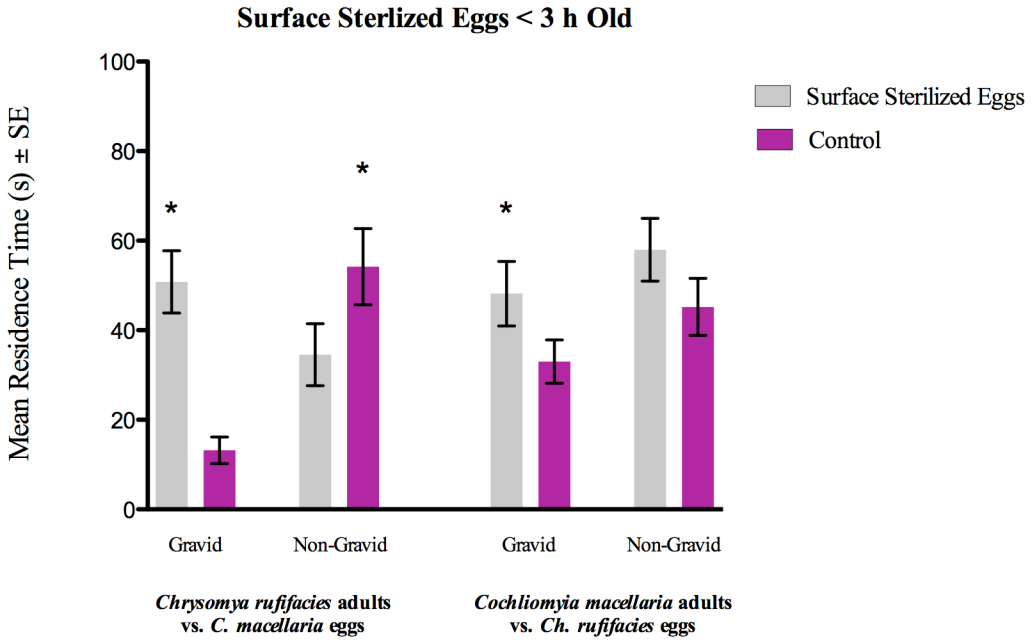


Fig 4.8. Mean residence time (mean ± SE) of adult flies presented with surface-sterilized heterospecific eggs < 3 h old in the dual choice olfactometer. * indicates significant ($P \leq 0.05$) difference in residence time.

Conspecific Response to Surface-Sterilized Eggs 3-6 h Old. Surface-sterilized eggs aged 3-6 h yielded insignificant first choice response data for developed females, while non-gravid females yielded significant first choice data in response to heterospecific eggs. Gravid *Ch. rufifacies* ($\chi^2 = 3.60$; df = 1; $P = 0.06$) and non-gravid (χ^2 (1, $N = 50$) = 0.50; $P = 0.48$) exposed to conspecific surface-sterilized eggs were not significantly attracted to the eggs treatment over the control. Similarly, *C. macellaria* adults presented with conspecific surface-sterilized eggs did not show significant first choice response for either fully developed females ($\chi^2 = 1.59$; df = 1; $P = 0.21$) or non-gravid females (χ^2 = 0.53; df = 1; $P = 0.47$) (Fig. 4.9).

Wilcoxon-rank-sum tests and subsequent Monte Carlo simulations analyzing

residence time data indicated significant differences in fly response to surface-sterilized eggs based on ovarian status (Fig. 4.10). Gravid *Ch. rufifacies* (z = -2.17; P = 0.01) and non-gravid (z = -1.78; P = 0.03) exposed to conspecific surface-sterilized eggs spent significantly more time in olfactometer arms associated with eggs. *Cochliomyia macellaria* adults presented with conspecific surface-sterilized fresh eggs did not show significant residence time response for either fully developed adults (z = -0.085; P = 0.471) or non-gravid females (z = -1.138; P = 0.129) (Fig. 4.9).

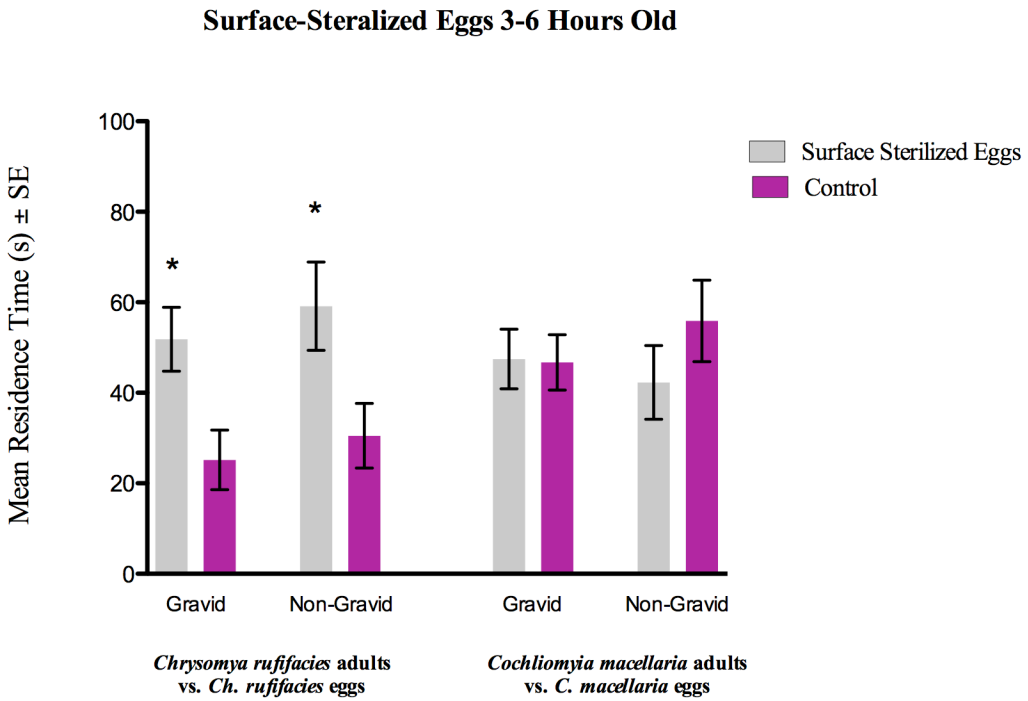


Fig 4.9. Mean residence time (mean ± SE) of adult flies presented with surface-sterilized conspecific eggs 3-6 h old in the dual choice olfactometer. * indicates significant ($P \leq 0.05$) difference in residence time.

Heterospecific Response to Surface-Sterilized Eggs 3-6 h Old. *Chrysomya rufifacies* adults presented with surface-sterilized *C. macellaria* eggs did not show significant first

choice response for fully developed females ($\chi^2 = 0.095$; df = 1; $P = 0.758$), but non-gravid females were significantly attracted to the eggs over the control ($\chi^2 = 6.533$; df = 1; $P = 0.011$). *Cochliomyia macellaria* adults presented with heterospecific surface-sterilized eggs showed no significant first choice data for fully developed females ($\chi^2 = 2.174$; df = 1; $P = 0.140$), but non-gravid females were significantly attracted to the control over the surface-sterilized eggs ($\chi^2 = 5.77$; df = 1; $P = 0.02$) (Fig. 4.10).

Chrysomya rufifacies fully developed females presented with surface-sterilized fresh *C. macellaria* eggs showed no significant difference in residence time ($z = -0.63$, $P = 0.261$), but pre-gravid females spent significantly more time in arms associated with eggs over those associated with the control ($z = -2.23$; $P = 0.01$). Gravid *C. macellaria* ($z = -3.52$; $P < 0.0001$) and non-gravid ($z = -2.165$; $P = 0.01$) presented with surface-sterilized heterospecific eggs spent significantly more time in arms associated with the control over those associated with eggs (Fig. 4.10).

Conspecific Response to Microbial Isolates from Eggs < 3 h Old. Microbial isolates from fresh eggs (< 3 h old) yielded predominantly non-significant first choice response data, with one exception. Gravid *Ch. rufifacies* adults ($\chi^2 = 1.09$; df = 1; $P = 0.30$) and non-gravid ($\chi^2 = 0.88$; df = 1; $P = 0.35$) exposed to conspecific microbes were not significantly attracted to the microbes over the control. *Cochliomyia macellaria* adults presented with conspecific microbes did not show significant first choice response for either gravid females ($\chi^2 = 2.941$; df = 1; $P = 0.07$) or non-gravid females ($\chi^2 = 0.33$; df = 1; $P = 0.70$) (Fig. 4.11).

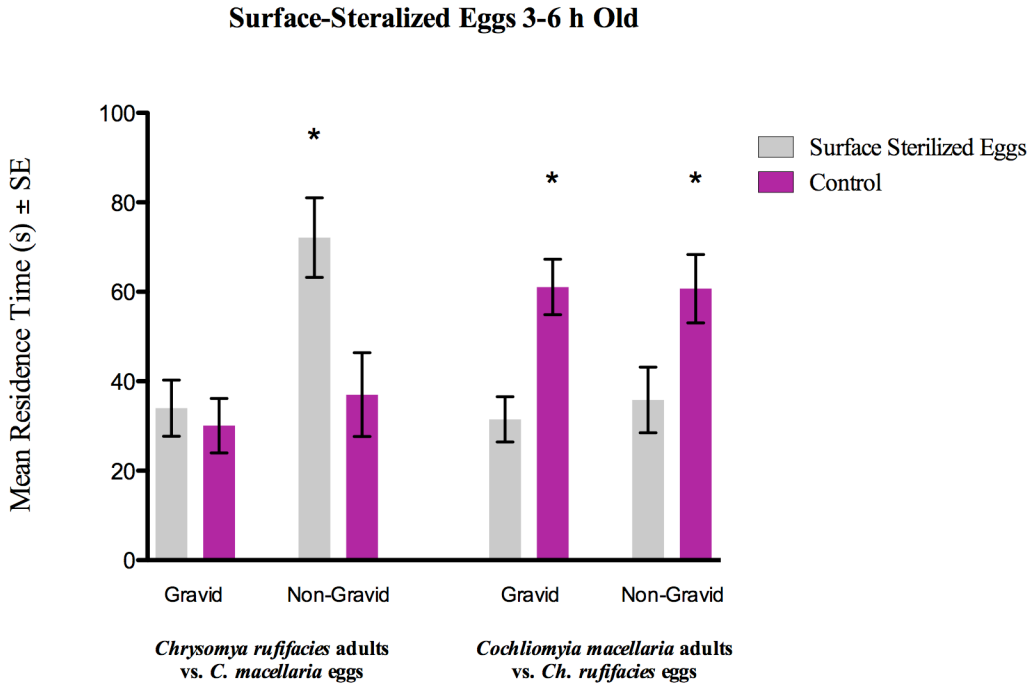


Fig 4.10. Mean residence time (mean ± SE) of adult flies presented with surface-sterilized heterospecific eggs 3-6 h old in the dual choice olfactometer. * indicates significant ($P \leq 0.05$) difference in residence time.

Wilcoxon-rank-sum tests and subsequent Monte Carlo simulations analyzing residence time data indicated significant differences in fly response to microbes based on ovarian status (Fig. 4.12). Gravid *Chrysomya rufifacies* females exposed to conspecific microbes spent significantly more time in olfactometer arms associated with microbes over the control (z = -1.74; P = 0.04). Non-gravid females, however, spent significantly more time in arms associated with the control over those associated with conspecific microbes (z = -2.51; P = 0.01). *Cochliomyia macellaria* adults presented with conspecific microbes did not show significant residence time response for gravid adults (z = -1.54; P = 0.06), but non-gravid females (z = -2.21; P = 0.01) spent significantly more time in areas associated with microbes over the control (Fig. 4.11).

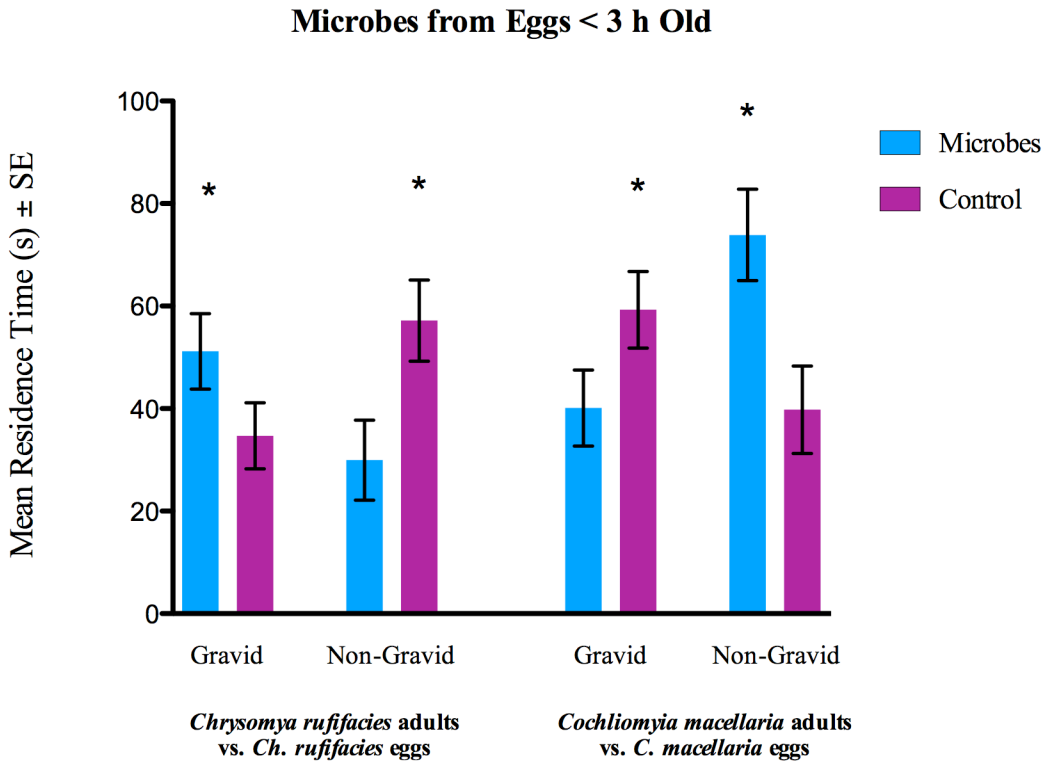


Fig 4.11. Mean residence time (mean ± SE) of adult flies presented with microbes from conspecific eggs < 3 h old in the dual choice olfactometer. * indicates significant ($P \leq 0.05$) difference in residence time.

Heterospecific Response to Microbial Isolates from Eggs < 3 h Old. Gravid *Ch. rufifacies* were significantly attracted to heterospecific microbes from fresh eggs (χ^2 = 27.92; df = 1; $P < 0.0001$). Non-gravid females, however, were not significantly attracted to the microbes ($\chi^2 = 0.12$; df = 1; $P = 0.73$). Gravid females ($\chi^2 = 0.86$; df = 1; $P = 0.36$) or non-gravid females ($\chi^2 = 0.95$; df = 1; $P = 0.33$) did not show a significant first choice to heterospecific microbes (Fig. 4.12).

Chrysomya rufifacies gravid females presented with heterospecific microbes spent significantly more time in areas associated with those microbes (z = -6.91; $P <$

0.0001), while non-gravid females showed no significant difference in residence time (z = -0.61; P = 0.28). *Cochliomyia macellaria* gravid females presented with heterospecific microbes spent significantly more time in areas associated with microbes (z = -2.48; P = 0.01), while non-gravid females showed no significant difference in residence time (z = -0.84; P = 0.20) (Fig. 4.12).

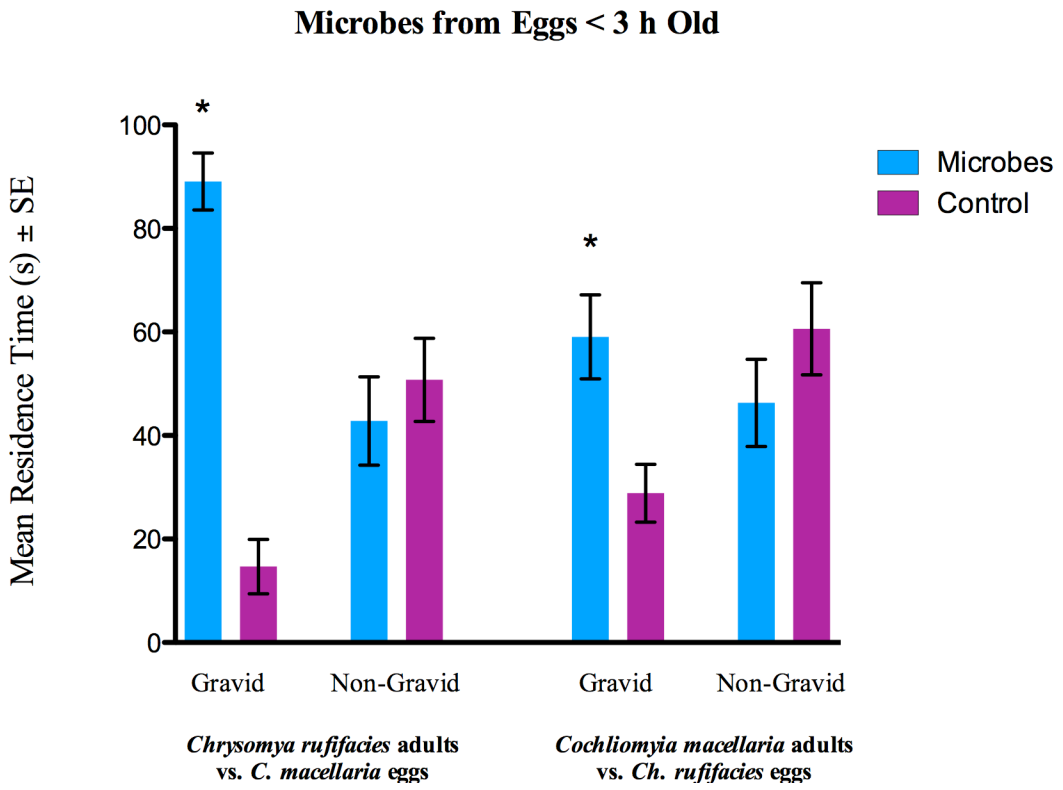


Fig 4.12. Mean residence time (mean ± SE) of adult flies presented with microbes from heterospecific eggs < 3 h old in the dual choice olfactometer. * indicates significant ($P \leq 0.05$) difference in residence time.

Conspecific Response to Microbial Isolates from Eggs 3-6 h Old. Microbial isolates from eggs aged 3-6 h yielded significant first choice response data based on ovarian

status. *Chrysomya rufifacies* gravid females were significantly attracted to conspecific microbes ($\chi^2 = 3.90$; df = 1; $P = 0.05$), while non-gravid females were not significantly attracted to conspecific microbes over the control ($\chi^2 = 2.29$; df = 1; $P = 0.13$). Conversely, gravid *C. macellaria* females were not significantly attracted to conspecific microbes ($\chi^2 = 0.13$; df = 1; $P = 0.72$), while non-gravid females were significantly attracted to conspecific microbes over the control ($\chi^2 = 7.20$; df = 1; $P = 0.01$) (Fig. 4.13).

Wilcoxon-rank-sum tests and subsequent Monte Carlo simulations of residence time data indicated significant differences in fly response to treatments based on ovarian status (Fig. 4.14). *Chrysomya rufifacies* gravid ($z = -2.57$; $P = 0.01$) and non-gravid females ($z = -2.32$; $P = 0.02$) exposed to conspecific microbes spent significantly more time in olfactometer areas associated with the control over arms associated with microbes. *Cochliomyia macellaria* gravid ($z = -0.03$; $P = 0.98$) or non-gravid females ($z = -2.79$; $P = 0.07$) presented with conspecific microbes did not show significant residence time response (Fig. 4.13).

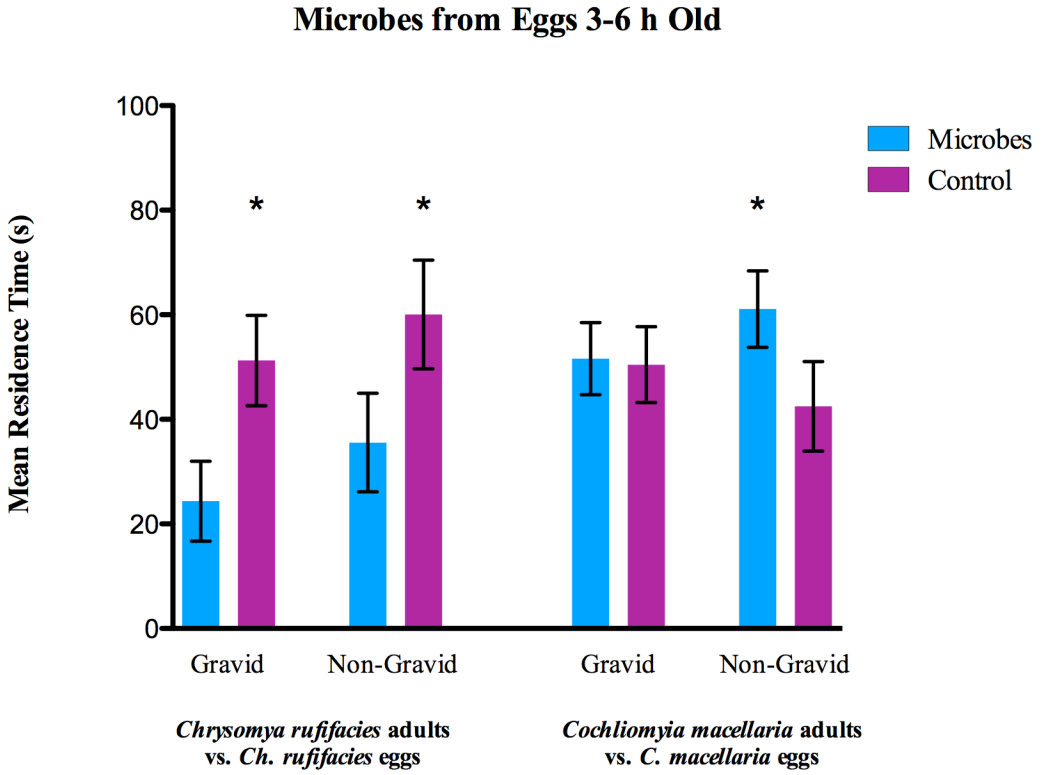


Fig 4.13. Mean residence time (mean ± SE) of adult flies presented with microbes from conspecific eggs 3-6 h old in the dual choice olfactometer. * indicates significant ($P \leq 0.05$) difference in residence time.

Heterospecific Response to Microbial Isolates from Eggs 3-6 h Old. *Chrysomya rufifacies* adults presented with microbes from *C. macellaria* eggs did not show significant first choice response for either gravid (χ^2 (1, $N = 50$) = 0.333, $P = 0.564$) or non-gravid females ($\chi^2 = 0.36$; df = 1; $P = 0.55$). Similarly, *C. macellaria* adults presented with heterospecific microbes showed no significant first choice data for gravid ($\chi^2 = 0.12$; df = 1; $P = 0.73$) or non-gravid females ($\chi^2 = 0.60$; df = 1; $P = 0.44$) (Fig. 4.14).

Chrysomya rufifacies gravid ($z = -0.48$; $P = 0.64$) and non-gravid females ($z =$

-2.00; df = 1; P = 0.20) presented with heterospecific microbes showed no significant difference in residence time. Finally, *C. macellaria* gravid (z = -0.05; P = 0.96) and non-gravid females (z = -0.00; P = 1.00) presented with heterospecific microbes showed no significant resident time response (Fig. 4.14).

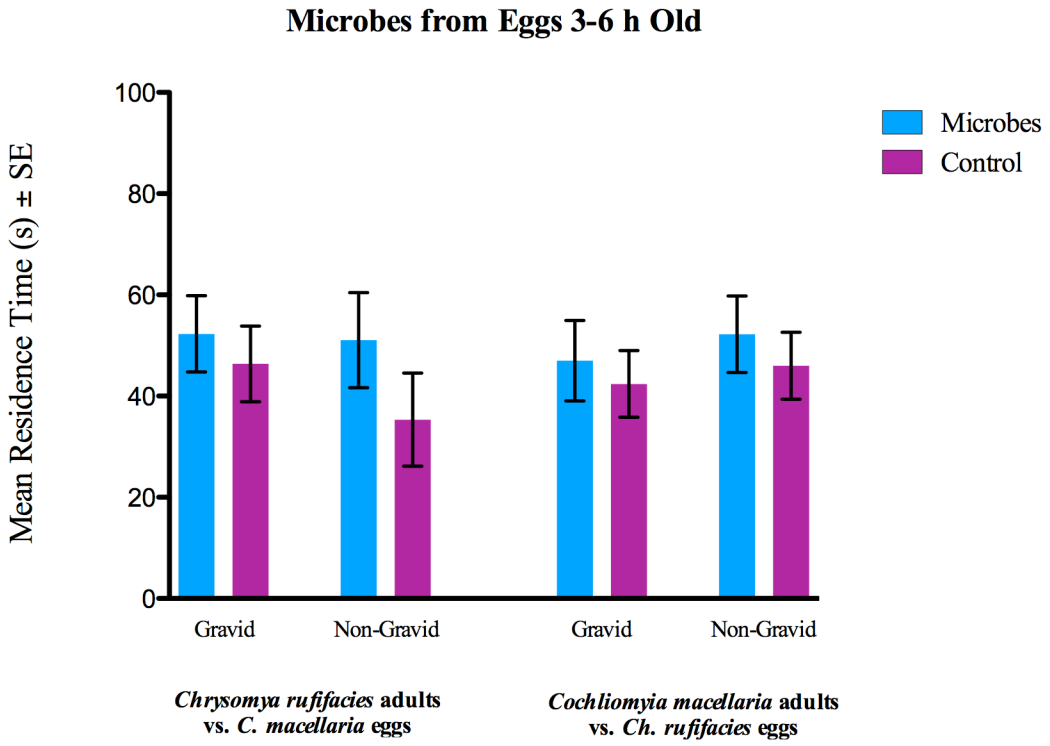


Fig 4.14. Mean residence time (mean ± SE) of adult flies presented with microbes from heterospecific eggs 3-6 h old in the dual choice olfactometer. * indicates significant ($P \leq 0.05$) difference in residence time.

Relative Abundance of Bacterial Genera on *Cochliomyia macellaria* Eggs. Sixty-eight bacterial genera (exhibiting a ≥ 95% similarity with reference samples) were detected on all egg stages of *C. macellaria* (Table B2). The total number of bacterial genera detected on eggs < 3 h old was 39; on eggs 3-6 h old was 37; and on eggs 6-9 h

old was 29. The highest percentage of bacterial genera found on eggs < 3 h old was *Lactobacillus sp.* (26.45%), followed by *Vagococcus sp.* (16.53%), *Morganella sp* (9.71%), *Carnobacterium sp.* (9.13%), *Providencia sp.* (4.76%), *Pseudochrobatrum* sp. (4.22%), *Leuconostoc sp.* (3.36%), and *Enterococcus* (1.98%). All other species made up < 1% of the relative bacterial abundance each (Fig. 4.15). Bacterial genera that were not identified were categorized as "Unclassified," and comprised 17.57% of the species found on eggs < 3 h old.

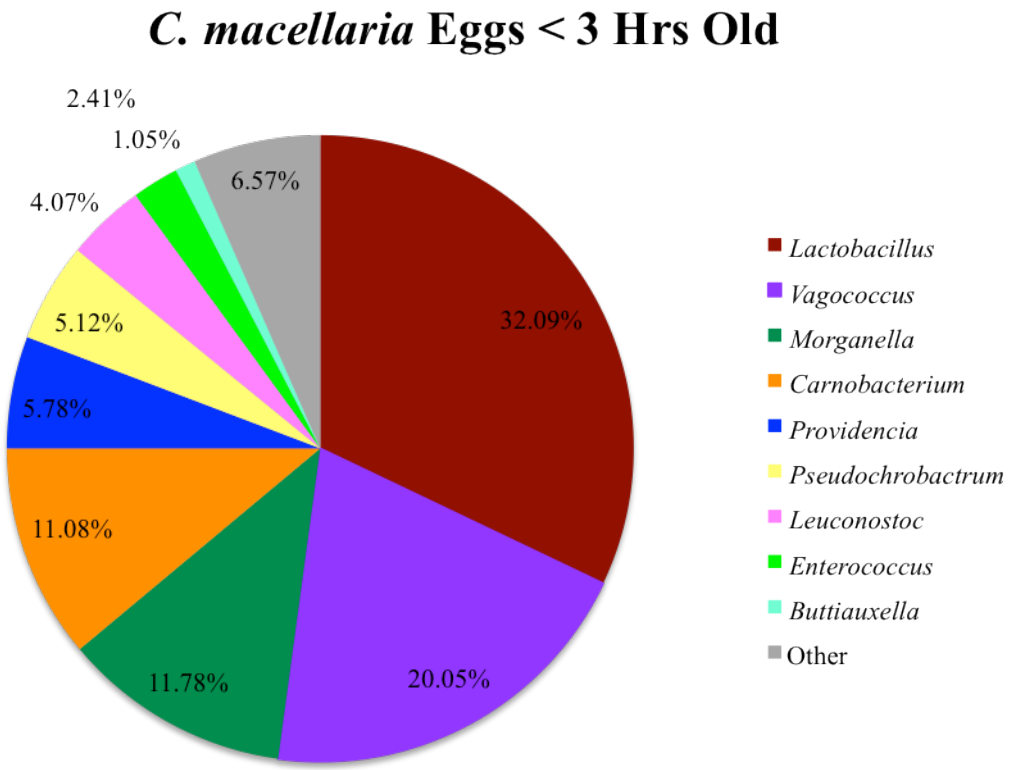


Fig 4.15. Relative abundance of bacterial genera by egg age on *Cochliomyia macellaria* eggs < 3 h old. Values below 1% were grouped as "Other" with total value of 6.57%.

The highest percentage of bacterial genera found on eggs 3-6 h old was *Escherichia* sp. (17.38%), followed by *Lactobacillus sp.* (10.91%), *Kurthia sp.* (8.31%), *Staphylococcus sp.* (7.63%), *Providencia sp.* (7.18%), *Lactococcus sp.* (1.30%), and *Yaniella sp.* (1.66%). Unclassified bacteria made up 37.90% of the species found on eggs 3-6 h old. All other species made up < 1% of the relative bacterial abundance each (Fig. 4.16).

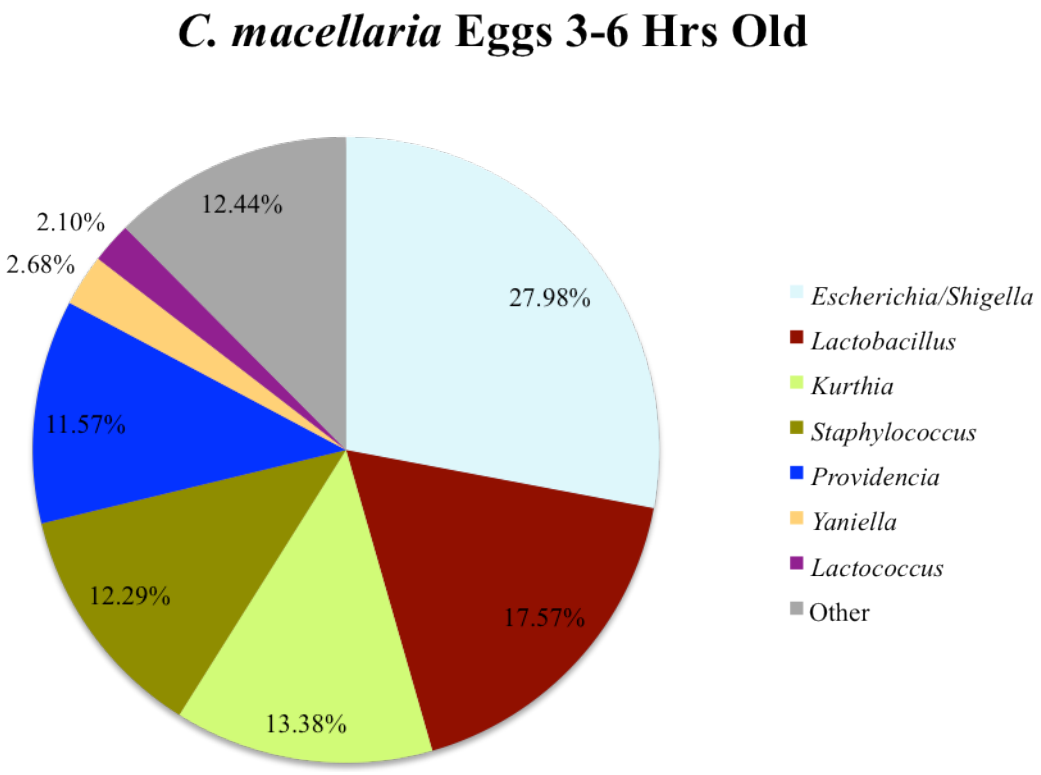


Fig 4.16. Relative abundance of bacterial genera by egg age on *Cochliomyia macellaria* eggs 3-6 h old. Values below 1% were grouped as "Other" with total value of 12.44%.

The highest percentage of bacterial genera found on eggs 6-9 h old was *Lactobacillus sp.* (54.81%), followed by *Carnobacterium sp.* (12.89%), *Vagococcus sp.* (6.52%), *Leuconostoc sp.* (3.78%), and *Providencia sp.* (1.83%). Unclassified bacteria made up

13.62% of the species found on eggs 6-9 h old. All other species made up < 1% of the relative bacterial abundance each (Fig. 4.17).

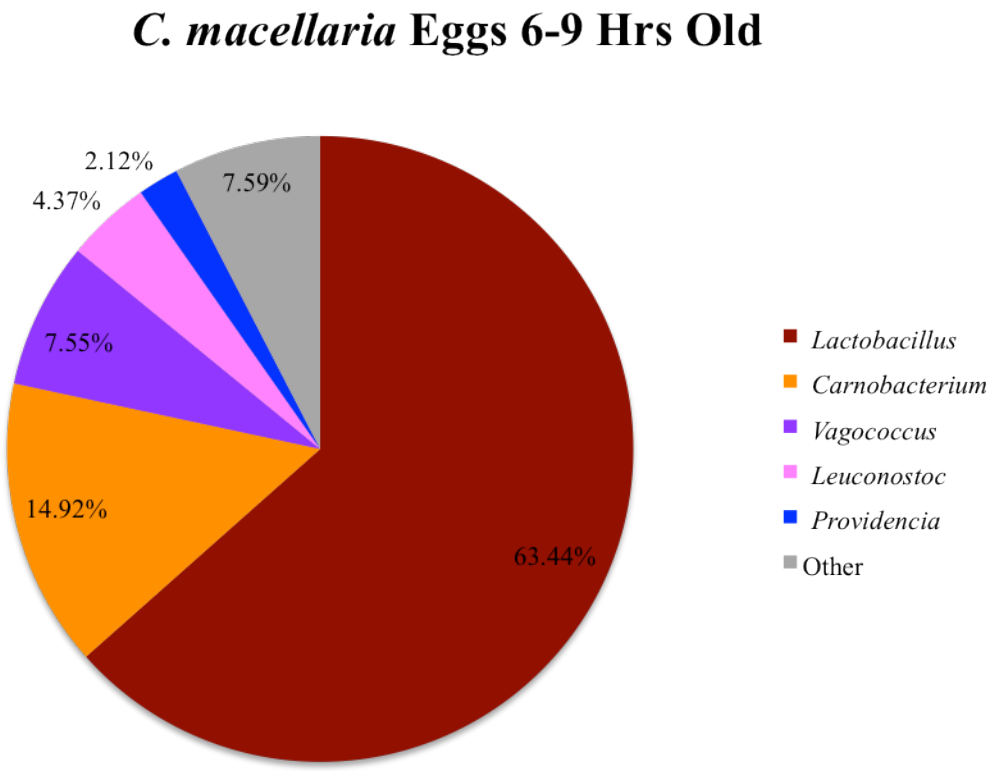


Fig 4.17. Relative abundance of bacterial genera by egg age on *Cochliomyia macellaria* eggs 6-9 h old. Values below 1% were grouped as "Other" with total value of 7.59%.

Relative Abundance of Bacterial Genera on *Chrysomya rufifacies* Eggs. Total number of bacterial genera detected on eggs < 3 h old was 30; on eggs 3-6 h old was 19; and on eggs 6-9 h old was 26. The highest percentage of bacteria found on eggs < 3 h old was *Lactobacillus sp*. (45.69%), followed by *Vagococcus sp.* (19.05%), *Lactococcus sp.* (12.57%), *Staphylococcus sp.* (2.22%), *Delftia sp.* (2.00%), and *Sphingobacterium sp.* (1.15%). All other species made up < 1% of the relative bacterial abundance each

(Fig. 4.18). Bacterial genera that were unable to be identified by pyrosequencing methods were categorized as "Unclassified," and made up 12.48% of the species found on eggs < 3 h old.

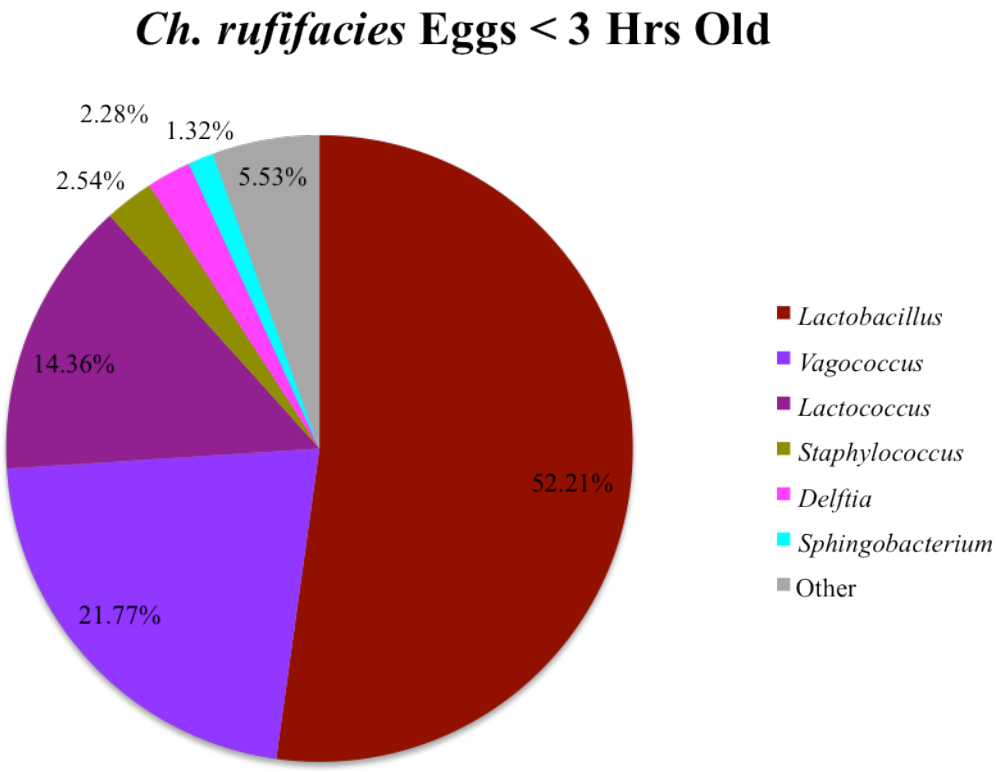


Fig 4.18. Relative abundance of bacterial genera by egg age on *Chrysomya rufifacies* eggs < 3 h old. Values below 1% were grouped as "Other" with total value of 5.53%.

The highest percentage of bacterial genera found on eggs 3-6 h old was *Lactococcus sp.* (42.07%), followed by *Lactobacillus sp* (23.83%), *Myroides sp.* (15.99%), *Vagococcus sp.* (4.86%), *Providencia sp.* (1.09%), and *Staphylococcus sp.* (1.03%). Unclassified bacteria made up 7.66% of the species found on eggs 3-6 h old. All other species made up < 1% of the relative bacterial abundance each (Fig. 4.19).

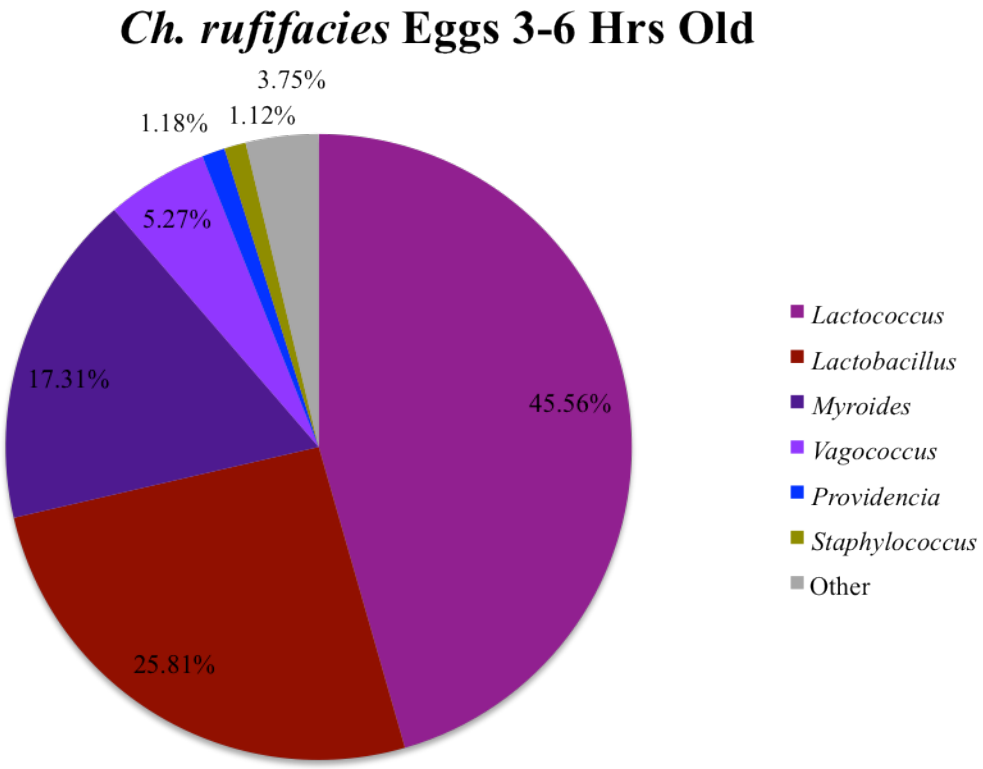


Fig 4.19. Relative abundance of bacterial genera by egg age on *Chrysomya rufifacies* eggs 3-6 h old. Values below 1% were grouped as "Other" with total value of 3.75%.

The highest percentage of bacterial genera found on eggs 6-9 h old was *Lactococcus sp.* (48.35%), followed by *Lactobacillus sp.* (15.35%), *Vagococcus sp.* (9.57%), *Providencia sp.* (4.75%), *Staphylococcus sp.* (3.37%), *Ignatzschineria sp.* (1.89%), and *Morganella sp.* (1.19%). Unclassified bacteria made up 11.42% of the species found on eggs 6-9 h old. All other species made up < 1% of the relative bacterial abundance each (Fig. 4.20).

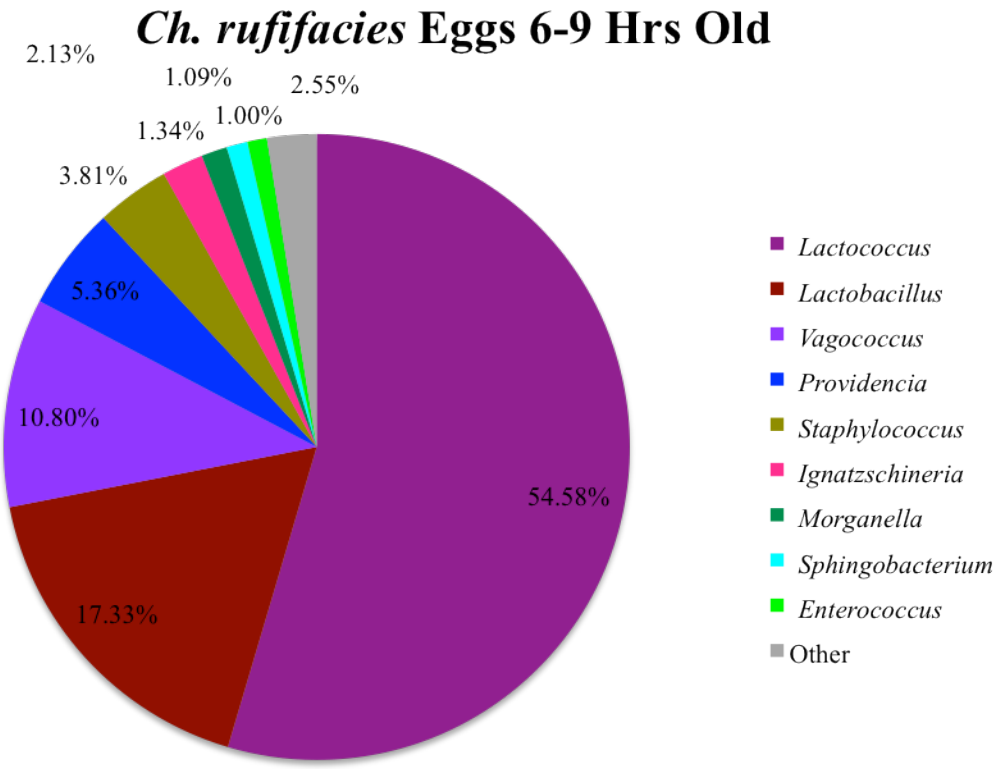


Fig 4.20. Relative abundance of bacterial genera by egg age on *Chrysomya rufifacies* eggs 6-9 h old. Values below 1% were grouped as "Other" with total value of 3.75%.

Discussion

First response and residence time were recorded in these experiments. First response appears to be much less informative than residence time. While significance was achieved in the first choice analyses of primarily *Ch. rufifacies* adults, observed activity in the olfactometer indicated a need for acclamation to the environment before an "informed" choice could be made. Comparison of first choice and residence time data confirms these observations, showing insignificant first choice responses and significant residence time responses in most of the experiments. Most adults spent the initial 30 s of exposure in the olfactometer exploring the environment before resting in an area associated with particular bait. Therefore, all discussion will be based on residence time responses.

Response to Egg Age. The age of both *C. macellaria* and *Ch. rufifacies* eggs influenced adult attraction, which was compounded by the physiological state and the sex of the adult. *Chrysomya rufifacies* tended to be attracted to both conspecfic and heterospecific eggs, while *C. macellaria* tended to be attracted to only conspecific eggs. *Chrysomya rufifacies* appeared to be more attracted to heterospecific eggs as they aged, rather than conspecific eggs. This may be due to the tendency of *Ch. rufifacies* to become cannibalistic in maggot masses. While group larval feeding may impart some benefit upon members of the maggot mass in the form of increased exodigestion efficacy (Fenton 1999, Rivers et al. 2011), the threat of cannibalism may select for maggot masses of similar aged larvae, rather than masses formed of larvae of varying ages. This pattern of adult regulation of cannibalistic larvae was observed in muscids by Lam et al.

(2007). *Musca domestica* (Linnaeus) (Diptera: Muscidae) females respond to conspecific, egg-assoicated cues that initially induce conspecific oviposition, and then inhibit oviposition after 24 h. This induction/inhibition evolution results in larval masses of similar ages, and protects younger larvae from cannabalism. The evolvoing cue in the muscid system is bacterial in origin, and eminates from bacteria found on the chorion of muscid eggs (Lam et al. 2007). It is not surprising, therefore, that *Ch. rufifacies* responds to conspecific eggs and egg-assoicated bacteria in a similar way.

Cochliomyia macellaria adults were attracted to conspecific eggs, but only as they reached 3-6 h old. This trend appeared to continue as *C. macellaria* eggs aged 6-9 h, and while significance at the 0.05 level was not technically reached ($P = 0.051$), the responses approached significance and may simply have failed to reach statistically significant levels due to low statistical power. This is counter intuitive, as field and lab observations reveal that *C. macellaria* oviposit in large groups (Brundage, unpublished), and therefore gravid females should be attracted to conspecific eggs of all ages. Olfactory responses to semiochemicals are an important part of the suite of behaviors (tactile, visual, olfactory, and thermal) resulting in blow flies exploiting resources (Hall 1995, Chapman 2003) However, olfaction may simply result in the activation of searching or oviposition behavior (Chapman 2003), and further stimuli are required to continue the behavior. Additionally, virgin female calliphorids are more reluctant to oviposit than mated females (Crystal 1967), and might therefore be responsive to odors attracting mated females to ovipositional sites (Hammack 1987) despite gravid status. It is possible that *C. macellaria* requires additional sensory input for attractancy, and that

the mating status of the females significantly affects olfactory response. This work illustrates conspecific egg attractancy that changes over time, but does not completely explain field observations.

Response to Surface-Sterilized Eggs. Analysis of fly response to egg-age experiments indicated that residence time in the olfactometer more informative than first choice response and residence time "choice" was made within 2 min of introduction into the olfactometer neck. Additionally, in order to allow greater efficiency, only the response of gravid females was recorded for the remaining experiments. Neither *C. macellaria* nor *Ch. rufifacies* females significantly responded to conspecific or heterospecific eggs 6-9 h old. Therefore, the remainder of this discussion focuses on gravid female flies exposed to eggs < 3 h or eggs 3-6 h old in the olfactometer for 2 min.

Response of gravid females of both species to eggs was reduced when the eggs were sterilized. Egg-associated microbes are known attractants in some fly species. Lam et al. (2007) found that gravid muscid flies were positively attracted to microbes cultured from the surface of conspecific eggs, although he did not identify which microbes were responsible for this attractancy. Lam's study showed that *M. domestica* laid 75% more eggs on oviposition sites dosed with egg-associated microbes over those without egg-assoicated microbes (Lam et al. 2007). This response in a species closely related to Calliphoridae, along with the reduced attractancy found in this experiment suggests that egg-assoicated microbes or their semiochemicals (message-bearing chemicals) are used as an oviposition cue.

The change in attraction and repellency of both fresh and aged eggs to gravid

adults due to surface-sterilization implies that surface-associated substances have an effect on adult behavior. During oviposition, eggs are laid in groups or clutches on the oviposition medium. The clutches are covered with a layer of glycoprotein that may prevent dehydration, and adheres the egg clutch to the substratum (Peterson 1991). The external surface of fly eggs are contaminated with bacteria (Mohd Masari et al. 2005), and this glycoprotein layer may be responsible for the adhesion of bacteria to the egg surface. While the glycoprotein layer itself or other unknown substances deposited during oviposition may be responsible for the observed patterns of adult attraction, the known attraction of microbial volatiles to adult Calliphoridae is a more likely explanation.

Microbes Associated with Eggs. Much more work has been done regarding the bacterial effect on attraction of blow flies to hosts (Fuller 1934a, Browne 1960, Eddy 1975, Emmens 1982). Emmens (1982) found *Pseudomonas sp.* degrades wool and produces sulphurous compounds, which attract female *Lucilia cuprina* (Wiedemann) (Diptera: Calliphoridae). Browne (1958) noted *Lucilia cuprina* increased oviposition in response to indole and ammonium carbonate, which are products of bacterial metabolism. Bovine blood inoculated with bacteria is significantly more attractive to the primary screwworm *Cochliomyia homnivorax* (Coquerell) (Diptera: Calliphoridae) than uninoculated blood (Eddy 1975, Chaudhury et al. 2010).

The change in microbial community may explain the change in attractancy behavior as eggs age. Both *Ch. rufifacies* and *C. macellaria* fresh eggs are dominated by *Lactobacillus* sp. As the eggs age to 3-6 h, *Lactococcus* takes over as the dominant

species on *Ch. rufifacies* eggs, while *Escherichia* takes over as the dominant species on *C. macellaria*. This change in dominance may be affecting the attractancy of *Ch. rufifacies* adults to eggs as they age. *Chrysomya rufifacies* is attracted to both conspecific and heterospecific fresh eggs, and *Lactobacillus* is the dominant species of bacterium found on those aged eggs. The females remain attracted to *C. macellaria* eggs as they age to 3-6 h, but lose the attractancy to conspecific eggs. Conspecific eggs become dominated by *Lactococcoccus* sp. during the 3-6 h range. It may be that *Lactococcus* is a repellant for *Ch. rufifacies.*

Cochliomyia macellaria show no such obvious patterns in their attractancy when compared to microbial change. Attraction to eggs in *C. macellaria* may be governed by a complex system of volatiles emanating from the egg itself, or from a combination of the bacterial community and the developing embryo. Investigation into the volatile production of both *C. macellaria* and *Ch. rufifacies* eggs and associated microbes is necessary to determine the extent to which these volatiles govern attractancy and repellency in *C. macellaria.*

While *Lactobacillus* and *Lactococcus* represent the dominant species on all eggs, and may therefore influcence adult behavior, this does not mean they are the only, or even the primary, species responsible for behavioral responses in Calliphoridae. In order to truly determine the effect of particular bacteria and bacterial community change on adult behavior, further work is necessary. Adult *Ch. rufifacies* and *C. macellaria* should both be presented with pure cultures of bacterial species, along with communities of bacterial species found on the eggs. The response of the adults to these treatments would

shed light on the actual affect bacterial volatiles have on the oviposition behavior of both species.

Conclusion. Eggs appear to be a major mediating factor in the oviposition timing of both *Ch. rufifacies* and *C. macellaria*. The attractancy changes over time, which implies the ability of ovipositing adults to assess egg age and mediate behavior in response to these cues. Additionally, the major sources of attractancy cues appear to be derived from egg-assoicated bacteria, which undergo substantial community change as the eggs age. Further, each species may be using these cues to differentiate between conspecific and heterospecific eggs, thereby directly affecting offspring fitness.

CHAPTER V

METHODS FOR EXTERNAL DISINFECTION OF BLOW FLY EGGS PRIOR TO USE IN WOUND DEBRIDEMENT THERAPY

Introduction

Maggot debridement therapy (MDT) is the use of necrophagic fly larvae to remove necrotic tissue and disinfect wounds (Sherman et al. 2000, Benbow 2006). This method efficiently removes devitalized tissue without damaging healthy cells (Vistnes 1981), while decreasing the bacterial load (Lerch 2003, Bexfield et al. 2008) and promoting tissue regeneration in chronic wounds (Livingston 1932). With the increase in antibiotic resistant strains of bacteria (Jaklic et al. 2008), MDT has enjoyed a upsurge in usage to augment conventional medical treatments all over the world (Sherman et al. 2000).

The use of biosurgical maggots spans centuries (Baer 1931, Mohd Masari et al. 2005). Mayan Indians cultivated these maggots to treat chronic wounds (Weil et al. 1933), while both the chief surgeon to Henri III and Napoleon's Surgeon-in-Chief used these "little surgeons" to prevent infection on the battlefield (Hobson 1931, Sherman et al. 2000). Work on the process in the early 20th century highlighted the necessity of surface sterilized larvae to prevent the introduction of pathogens into choric wounds (Baer 1931). Patients treated with unsterilized larvae had a 50% chance of contracting secondary infections (Baer 1931). Sterilization of the eggs and maggots prior to use reduced the likelihood of these infections occurring and led to the recommendation of

larval surface disinfection (Baer 1931).

Lucilia sericata (Meigen) (Diptera: Calliphoridae) is currently the primary necrophagic species used in MDT (Baer 1931, Weil et al. 1933, Sherman et al. 2000, Bexfield et al. 2008). *Lucilia sericata* larvae do not feed on healthy granulated tissue and are ideal for therapeutic uses (Weil et al. 1933). However, other blow fly species have been used throughout history, including *Lucilia cuprina* (Baer 1931, Fine 1934), *Lucilia Caesar* (Linnaeus) (Fine 1934) *Phormia regina* (Meigen) (Horn et al. 1976), *Calliphora vicina* (Robineau-Desvoidy) (Teich 1986), *Chrysomya rufifacies* (Macquart) (Sherman et al. 2000), and *Protophormia terraenovae* (Robineau-Desvoidy) (Diptera: Calliphoridae) (Sherman et al. 2000). *Cochliomyia macellaria* (Fabricius) and *Chrysomya megacephala* (Fabricius) (Diptera: Calliphoridae) are currently being investigated for efficacy as MDT agents (Fonsem, personal communication). Unfortunately, some of these species result in facultative myiasis and might not be appropriate for MDT.

Effective MDT relies on adequate aseptic technique to prevent the introduction of detrimental or pathogenic bacteria into wounds (Baer 1931). Practitioners of MDT tod prefer egg disinfection rather than maggot disinfection due to the higher rate of surface sterilization (Connell 1981, Sherman et al. 2000) and the higher survivorship of resulting larvae (Sherman et al. 2000). The external surface of fly eggs are contaminated with bacteria (Mohd Masari et al. 2005), resulting in newly hatched larvae becoming inoculated as well (Mackerras 1933a). Fine (1934) achieved an 80% sterilization rate using 10% Formalin immersion, and reported problems with sterilizing all instars of

maggots, (Fine 1934). Mohd Masari et al. (2005) reported similar results using 70% ethanol (EtOH) rinse and UV exposure, finding that surface sterilized maggots maintained sterility for only 24 h and had a survival rate of 20% (Mohd Masari et al. 2005). Comparatively, Connell (1981) determined surface sterilization of eggs resulted in contamination free eggs for 48 h after sterilization with a hatch rate of 75-90% (Connell 1981).

Studies on surface sterilization of dipteran eggs have been reported (Baer 1931, Simmons 1934, Connell 1980, Mohd Masari et al. 2005). However, measurement of the efficacy of different disinfection techniques in combination with the viability of the resulting eggs is lacking. Most reports detail the ultimate result of surface sterilization (Baer 1931, Mohd Masari et al. 2005) without quantifying the initial bacterial load of the eggs. Others report on the efficacy of sterilization without reporting the ultimate egg viability (Simmons 1934, Figueroa et al. 2007). To truly understand the effect of disinfectants on surface sterilization of dipteran eggs, the initial bacterial load must be taken into account along with the hatch rate of disinfected eggs.

A wide variety of methods used to sterilize dipteran eggs have been reported. Mercuric chloride (Baer 1931, Mackerras 1933b), Formalin (Simmons 1934), formaldehyde (H_2CO) (Horn et al. 1976, Mumcuoglu et al. 2001, Jaklic et al. 2008), Lysol® (Sherman and Tran 1995, Sherman 1996), 5% sodium hypochlorite (NaOCl) (Clorox® Bleach) (Mumcuoglu et al. 2001), ethanol (EtOH) (Brookes 1961), UV light (Mohd Masari et al. 2005), benzalkonium chloride (ADBAC) (Connell 1981) and 1% NaOCl (Teich 1986) are examples of techniques employed. The wide diversity of

chemicals used, and the sparse reporting of efficiency in conjunction with the subsequent hatch rate compelled the need to validate and standardize a procedure for future MDT practice.

The present study investigated egg disinfectant techniques previously reported in the scientific literature, which demonstrated efficient sterilization (≥80%) (Greenberg 1970, Sherman 1996) and (if reported) high hatch rates (≥70%) (Simmons 1934, Greenberg 1970, Connell 1981). These methods were tested on four species of Calliphoridae: *Cochliomyia macellaria*, *Chrysomya rufifacies*, *Lucilia sericata,* and *Lucilia cuprina*.

Material and Methods

Adult Fly Colony. Laboratory colonies of *Ch. rufifacies* and *C. macellaria* used in this study were collected in Brazos County, Texas area during spring and summer of 2009 and 2010, and were maintained by Adrienne Brundage (Texas A&M University, College Station, TX). *L. sericata* and *L. cuprina* were originally isolated from carrion in Los Angeles County, California, and were maintained by Dr. Aaron Tarone (Texas A&M University, College Station, TX). Fly larvae were reared on 50 g of bovine liver in 950 ml glass jars (Ball Corporation, Broomfield, CO) covered with 125 mm x 125 mm square Wypall™ L40 wipers (Kimberly-Clark, Irving, TX) tops and held at 27^0C ± 1^0C, 60% RH, and a 12:12 (L:D) photoperiod. Adult flies were maintained in 299 cm^3 aluminum cages (Bioquip Products, Rancho Dominques, CA) and provided reverse osmosis (RO) water and sugar *ad libitum*. Bovine liver was provided as a protein meal

two ds after eclosion. Eggs were obtained for experiments by placing 10 g of fresh bovine liver as an oviposition medium in appropriate fly colonies for 3 h. After which eggs were physically removed from the oviposition medium and deagglutinated before sterilization.

Deagglutination of Eggs. Deagglutination was performed on all eggs prior to treatments to ensure maximum treatment efficiency (Simmons 1934). Freshly laid eggs were removed from the oviposition medium and placed on 25 cm^2 wet, black, fine mesh cloth. A paper towel moistened with RO H_2O was folded over the cloth and allowed to sit for five min. Eggs were physically manipulated with a 10 mm synthetic fiber paintbrush (Loew-Cornell, Rye, NY) to facilitate separation

Immersion Fatality. Ability of each fly species to tolerate immersion in water was determined by a series of egg immersion experiments. Eggs of each species were tested for their ability to tolerate immersion in RO H_2O for 1, 3, 5, 7, and 10 min. Treatments consisted of 35 samples of 10 deagglutinated eggs each. Deagglutinated eggs that were not immersed in water were used as a control. Each sample was placed on a 35 mm^2 black cloth square, and the cloth folded into quarters to form a packet to prevent displacement of eggs while under water. Each sample was immersed in 10 ml RO for the appropriate amount of time then removed, drained on paper towels, and placed on 10 ml tryptic soy agar (TSA; Difco, Sparks, MD) in a sterile 30 ml plastic cup (Bio-Serv, Frenchtown, NJ) at 27°C. After 24 h incubation, the hatch rate was determined. The species most sensitive to immersion was utilized as the sentinel species to determine the efficacy of the disinfection techniques.

Disinfecting Agents. Ten treatments from the literature were tested based on their reported high sterilization efficiency and hatch rate. Two techniques, immersion or rinse was used (as defined below). Immersions utilized 5 or 10 % formalin for 5 min; 3% Lysol® for 5 or 10 min; 5% H_2CO for 5 min; and 5% NaOCl followed by 5% H_2CO for 5 min each. Rinses utilized 10 cc 70% EtOH; 30 cc 1% NaOCl. Combinations included ADBAC immersion for 10 min followed by 10 cc 70% ethanol rinse; and 95% EtOH evaporation for 5 min followed by SorGon® immersion for 5 min. Both 95% EtOH and 70% EtOH were diluted to working concentrations with sterile dH_2O. SporGon® and Lysol® were used in the commercially available formulations.

Each treatment consisted of 10 samples of 10 eggs per sample. Eggs were placed on a sterile 13 mm, 20 μm nylon membrane filter (GE Osmonics, Minnetonka, MN) and enclosed within a sterile 13 mm polycarbonate luer-lock filter holder (Cole-Parmer, Court Vernon Hills, IL). For immersion treatments, 5 ml of the appropriate disinfectant was loaded into a sterile 10 ml polypropylene luer-lock syringe (Chemglass, Vineland, NJ), and a volume of 2.5 ml was gently filtered to fill the filter holder with disinfectant. The eggs were thus immersed in the disinfectant within the filter and incubated at room temperature for the appropriate time, and then the full volume was gently evacuated through the filter. For rinse treatments, 10 cc of the disinfectant was loaded into the sterile luer-lock syringe, and immediately evacuated through the filter at a rate of 0.5 cc per second. After each immersion or rinse treatment, 20 cc of sterile Pringle's insect saline (mM concentration in deionized water: NaCl 154, KCl 2.68, $CaCl_2$ 1.8, L-glucose 22.2) (Pringle 1938) was filtered through the apparatus to rinse the eggs of residual

disinfectant. To collect a sample of the bacteria present on the external egg surface (PRE-wash), deagglutinated eggs were immersed in 2 ml of tryptic soy broth (TSB; Difco, Sparks, MD) at room temperature for 3 min. Eggs were removed and observed for hatch rate. PRE-wash bacterial load, in CFU (colony forming units), was determined by serial dilution onto blood agar plates and incubation at 37°C for 18 h.

For each disinfection protocol, the residual bacteria remaining after treatment (POST-wash) was sampled by incubating the treated eggs in 2 ml of TSB at room temperature for 3 min. The eggs were then removed and the bacterial load was enumerated by serial dilution onto blood agar plates (BVA Scientific, San Antonio, TX) followed by incubation for 18-24 h at 37°C. An aliquot of PRE-wash and POST-wash TSB was enriched by overnight incubation at 37°C, to ensure bacterial detection below the plating threshold of ~10 CFU. These enrichments were plated on blood agar and incubated for 18-24 h at 37°C. Following each disinfection and POST-wash protocol, eggs were placed in 30 ml clear plastic cups (Bio-Serv, Frenchtown, NJ) containing 10 ml TSA and incubated at 27°C. The egg hatch rate was determined after 24 h.

To visualize the effect of treatment protocols on the egg chorion, additional egg samples were subjected to treatment protocols and stained to visualize the chorion. Ten eggs were placed on filters and treated in conjunction with other egg samples, but did not undergo PRE- or POST-wash treatments. These eggs were stained with potassium permanganate following Sukontason et al. (2004). Eggs were transferred into a glass petri dish using a small camel hairbrush and covered with 10 µl one percent potassium permanganate solution (Thermo Fisher Scientific Inc., Waltham, MA). Eggs were

soaked in this solution for one minute, after which excess potassium permanganate solution was removed using filter paper. Eggs were then dehydrated by passing them through 15, 70, 95% and absolute alcohol (each solution for one minute) and transferred into three drops of slide mounting medium (60% resin in xylene) (Bioquip Products, Rancho Dominques, CA). A cover slip was placed over the eggs, and each mounted, stained egg was examined under a light microscope.

Agitation Treatments. After initial disinfection treatments were analyzed, three treatments reported to be effective in other venues (Teich 1986, Sherman 1996, Crippen 2006) were selected for their potential as a surface disinfectant given an environment, which separated eggs to allow improved surface contact. Agitation or additional rinsing of the eggs was added to help mitigate the effects of agglutination during the disinfection process, which was common during NaOCl, EtOH, and SporGon treatments. This agglutination may lower access of the chemical to the entire surface area of all eggs. The treatments selected were SporGon®, 1% NaOCl, and 3% Lysol®. Either rinse or agitation in sterile insect saline was used as a control, as this insect saline neither significantly lowers the amount of bacterial contamination on eggs surfaces, nor lowers the total eclosion.

Each treatment consisted of 10 samples, with 10 eggs per sample and placed on filters as described above. For agitation treatments, 5 ml of appropriate disinfectant was loaded into a sterile luer-lock syringe, and 2.5 ml was forced through the filter. This action filled the filter holder and assured contact of the disinfectant with the eggs. Syringes and filters were then placed onto a Roto Shake-Genie (Scientific Industries,

Bohemia, NY, USA) at 10 RPM for 5 min. After, excess liquid was evacuated through the filter and discarded. For additional rinse treatments, three rinses of 10 cc of disinfectant was loaded into a sterile luer-lock syringe, and strained through the filter at a rate of 0.5 cc per second. After each treatment, 20 cc of sterile insect saline was loaded into a sterile luer-lock syringe and eggs were rinsed to remove residual disinfectant.

Additional Species Examined. Two treatments, disinfection by Lysol® 10 min soak and $_{H2CO}$, were chosen for analysis on the eggs of three additional species, *Ch. rufifacies*, *L. sericata* and *C. macellaria*. Treatment selection was based on, 1) results from initial and agitation treatments giving the highest rates of disinfection and egg hatch; and 2) common usage by a majority of commercial sterile maggot producing labs (Sherman, personal communication) as a preferred surface sterilization treatment.

Experimental Design and Statistics. Each experiment was replicated three times. Data to determine immersion fatality were analyzed using ANOVA and Tukey's HSD in SPSS 17 (SPSS Inc. 2010, Chicago, IL, USA).

Results

Immersion Fatality. *Chrysomya rufifacies* immersed for one minute (M = 91.21%, SD = 11.19), three minutes (M = 92.37%, SD = 7.77), five minutes (M = 91.17%, SD = 12.19), seven minutes (M = 92.66%, SD = 9.45) and ten minutes (M = 88.75%, SD = 15.68) showed no significant difference in percent of eggs hatch from control eggs (M = 92.29%, SD = 9.882) (P = 0.134). *Lucilia sericata* eggs immersed for one minute (M = 48.41%, SD 23.45), three minutes (M = 48.97%, SD = 27.44), five minutes (M =

49.14%, SD = 28.65), seven minutes (M = 52.17%, SD = 9.45) and ten minutes (M = 49.90%, SD = 27.79) also showed no significant difference in percent of eggs hatch from control eggs (M = 47.31%, SD = 23.45) (P = 0.8823). *Cochliomyia macellaria* exhibited a significantly lower ($P < 0.0001$) hatch rate than controls (M = 95.89%, SD = 9.93) only after a 10-minute immersion (M = 91.78%, SD = 13.27). *Lucilia cuprina* also exhibited a significantly lower ($P < 0.0001$) hatch rate than controls (M = 89.66%, SD = 11.07) after a five minute (M = 75.24%, SD = 29.34), seven minute (M = 74.38%, SD = 25.50) and ten minute (M = 76.30%, SD = 27.14) immersion. Because of the sensitivity to immersion demonstrated by *L. cuprina*, and the goal to develop an external disinfection procedure useful for all of the listed species, *L. cuprina* was utilized as a sentinel species to evaluate the various disinfectant treatments. (Fig. 5.1).

***Lucilia cuprina* Disinfection.** A comparison of external disinfection protocols demonstrated large variation in treatment efficacy, yet all protocols significantly lowered ($P < 0.0001$) the mean number of CFU present on egg surfaces when compared to the PRE-wash (M CFU: 5332, SEM: 2.76) (Table 5.1).

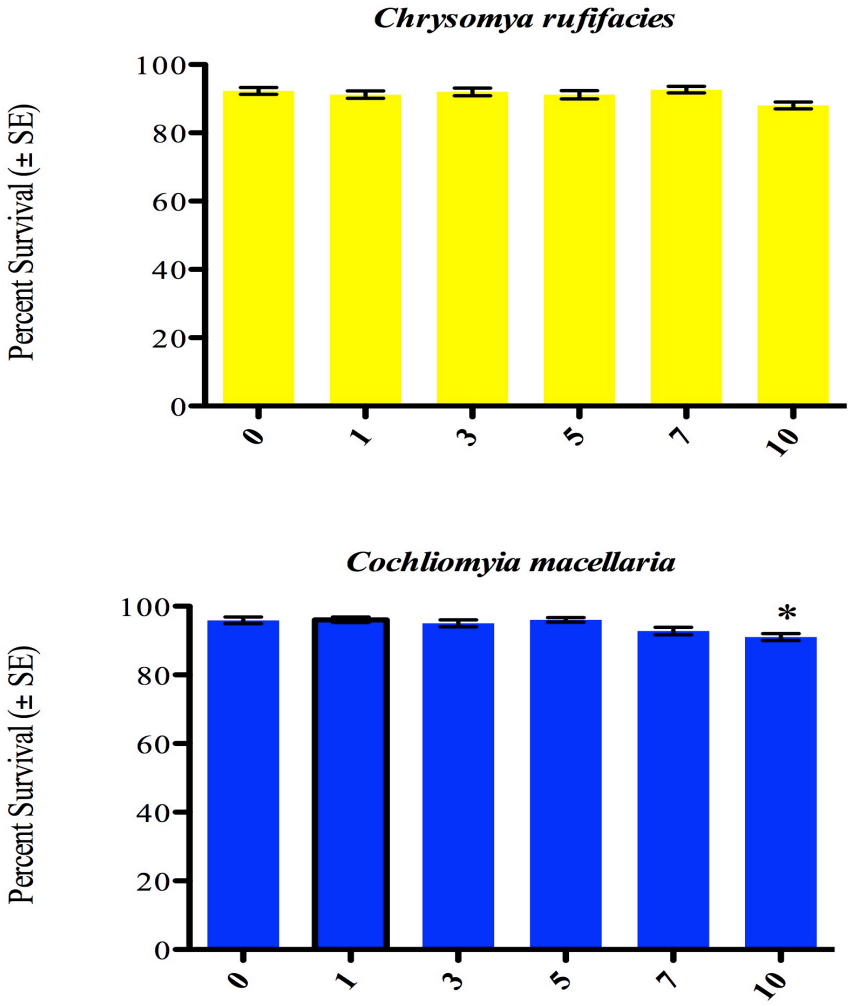


Fig 5.1. Percent egg survival after immersion in sterile RO water for 0 (control), 1, 3, 5, 7, or 10 minutes. Four species were tested for survival: *Chrysomya rufifacies, Cochliomyia macellaria, Lucilia sericata,* and *Lucilia cuprina.* * indicates significant ($P \leq 0.05$) differences from control. *Luicila cuprina* eggs were more sensitive to immersion than other species.

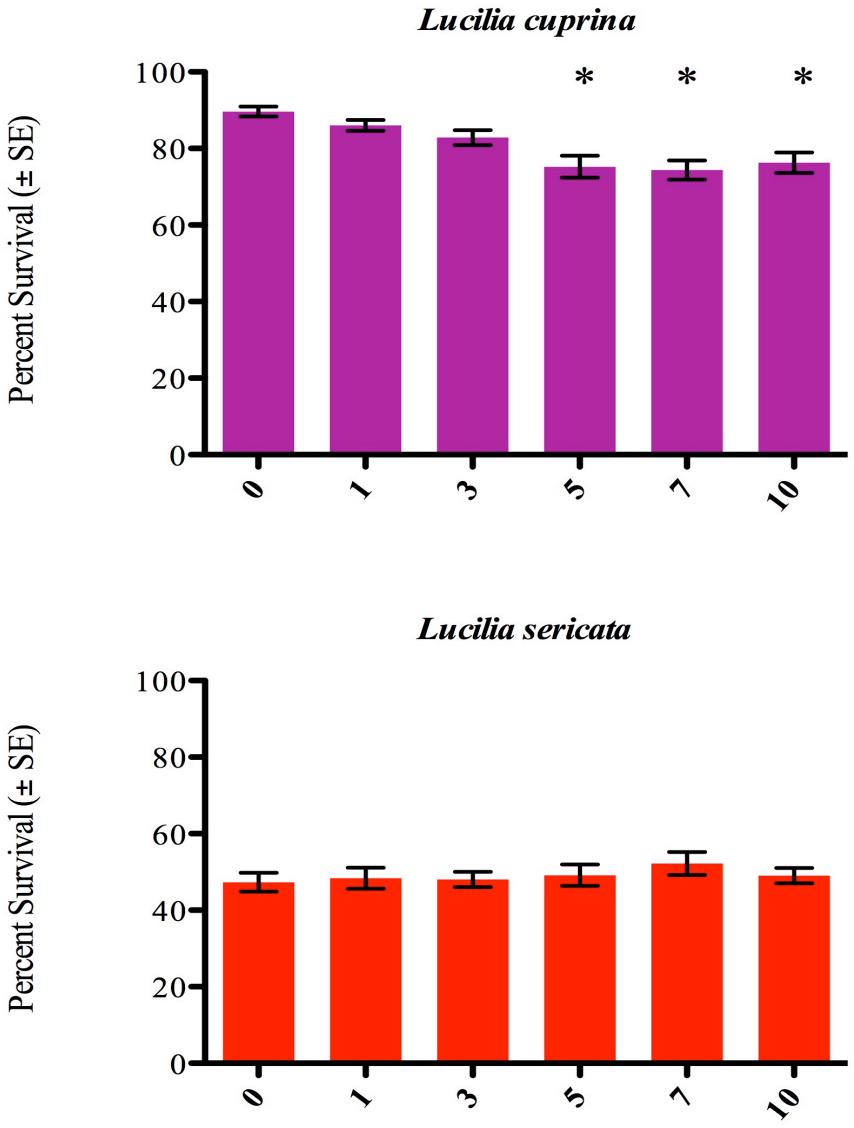


Fig 5.1 continued

Table 5.1. Comparison of the efficacy of surface disinfection protocols on *Lucilia cuprina* (Diptera: Calliphoridae) egg (n = 30)

Treatments	**Mean CFU ± SE**	**Enrichment (% Clean eggs ± SE)**
PRE-Wash	5332 ± 2.76	0
5% Formalin	987 ± 2.92	3.33 ±0.11
10% Formalin	126.34 ± 0.89	43.33 ± 0.82
3% Lysol, 5 minute immersion	15.31 ± 0.43	83.33 ± 0.28
3% Lysol, 10 minute immersion	0.00 ± 0.00	100 ± 0.00
Bleach + H2CO	20.48 ± 0.36	73.33 ± 0.69
H2CO	47.64 ± 0.68	66.67 ± 1.01
70% ETOH	515.72 ± 2.67	56.67 ± 0.74
1:50 Dilute bleach	85.89 ± 0.76	70.00 ± 0.55
Benzalkonium chloride	41.94 ± 0.95	33.33 ± 1.05
95% ETOH + SporGon	198.76 ± 2.49	23.33 ± 0.59

External disinfection using 5% formalin was the least efficacious of all treatments (*M* CFU = 987, SD 15.97) (Fig 5.2), resulting in only 3.33% of the samples yielding no residual bacteria after 24 h enrichment (Fig 5.3). Increasing the formalin to 10% decreased surface bacteria by 87% (*M* = 126.3, SD = 4.891) (Fig 5.2), and resulted in 43.33% of the samples harboring no residual bacteria after 24 h enrichment (Fig 5.3). Treatment with 70% EtOH was the second least effective treatment (*M* = 515.70 CFU, SD 14.62) (Fig 5.2), with only 53.33% of the samples yielding no residual bacteria after 24 h enrichment (Fig 5.3). Treatment with 95% EtOH followed by SporGon® immersion resulted in the third least-efficient method of surface disinfection (*M* CFU= 198.76, SD = 13.64) (Fig 5.2), and only 23% of the samples yielded no residual bacteria after 24 h enrichment (Fig 5.3). H_2CO immersion resulted in 63.33% of the samples yielding no residual bacteria after 24 h enrichment (Fig 5.3), and lowering the mean CFU by 99.67%

when compared to untreated controls (M = 47.64, SD = 3.73) (Fig 5.2). The addition of a prewash of bleach to the H_2CO treatment lowered the mean CFU an additional 53.33% (M = 20.48, SD = 1.94) (Fig 5.2) and resulted in 73.33% of the samples yielding no residual bacteria after 24 h enrichment (Fig 5.3). Diluting the bleach to 1% and applying it in a rinse increased mean CFU by 76.16% when compared to the bleach and H_2CO treatment (M = 85.89, SD = 4.19) (Fig 5.2) while resulting in 70.00% of the samples harboring no residual bacteria after 24 h enrichment (Fig 5.3). ADBAC performed similarly to H_2CO immersion (M = 41.94, SD = 5.18) (Fig 5.2) but only 33% of the samples yielded no residual bacteria after 24 h enrichment (Fig 5.3). Lysol® immersion yielded the highest disinfection efficacy over all. Immersion for 5 min was the second-most efficient disinfection method (M = 15.31; SD 2.34) (Fig 5.2), with 83% of the samples yielding no residual bacteria after 24 h enrichment (Fig 5.3). Increasing the Lysol® immersion time to 10 min increased the efficacy of surface disinfection (M = 0; SD = 0) (Fig 5.2); however 24 h enrichment of the samples showed only 96.67% of the samples harbored no residual bacteria (Fig. 5.3).

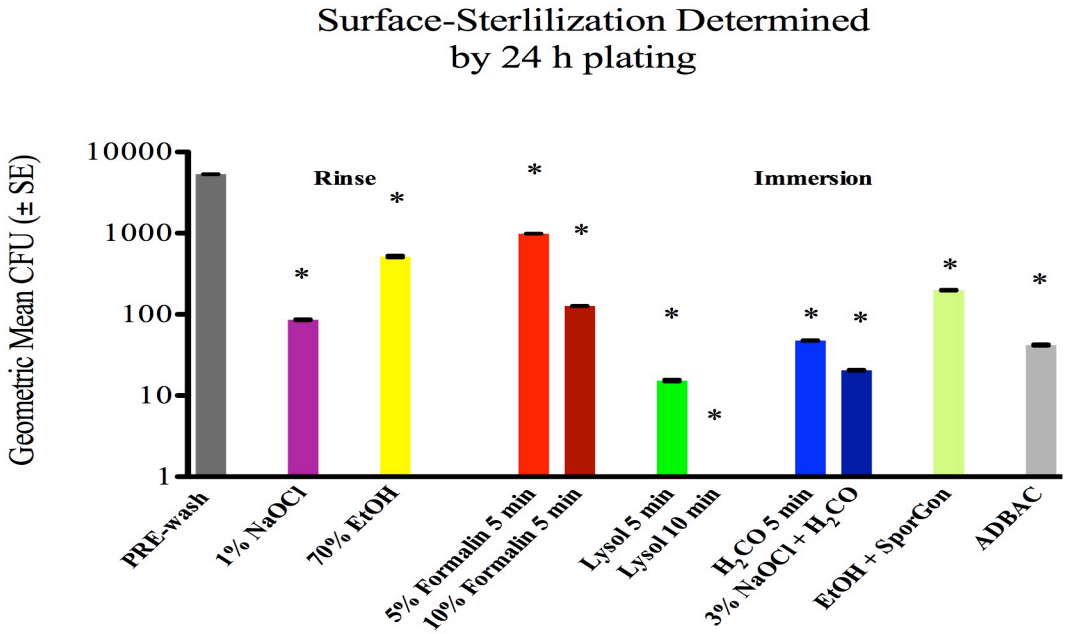


Fig 5.2. Geomean number of bacteria present on *Lucilia cuprina* egg surface (*N* = 30) post surface-sterilization determined by 24 h culture at 37 °C on blood agar. * indicates significant difference from PRE-wash mean CFU.

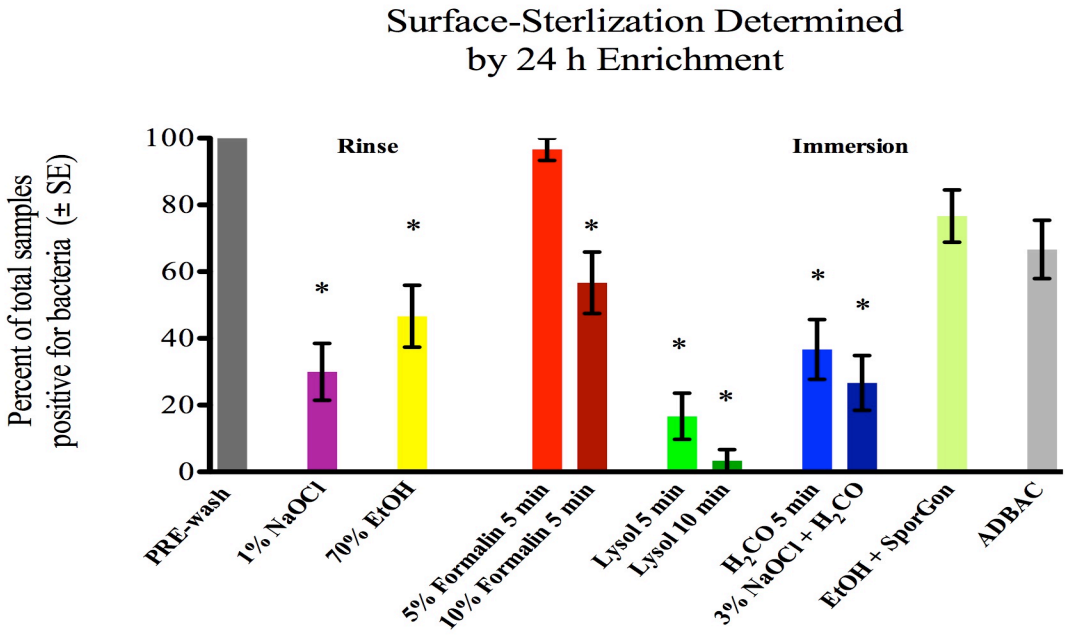


Fig 5.3. Percent of samples positive for bacteria after surface sterilization and 24 h enrichment in TSB at 37 °C. * indicates significant difference from PRE-wash mean positive samples.

***Lucilia cuprina* Egg Eclosion.** The mean egg eclosion for untreated *L. cuprina* eggs was 83.23% (Fig 5.4). Two treatments significantly ($P < 0.0001$) lowered the eclosion rates: immersion in H_2CO or in bleach and H_2CO. The mean eclosion was reduced to 59.84% ($P = 0.0018$) and 50.08% ($P = 0.0006$) following H_2CO or bleach and H_2CO immersion, respectively. No other treatments significantly decreased eclosion (Fig 5.4).

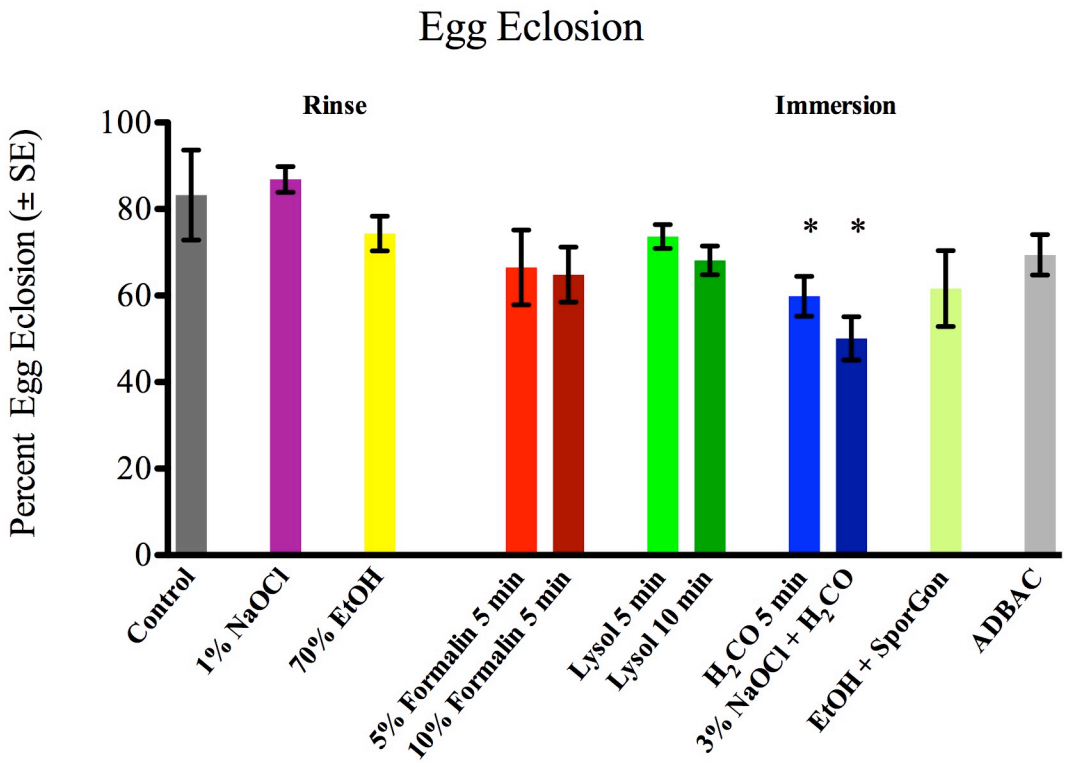


Fig 5.4. Mean number of eggs that successfully eclosed after surface-sterilization. * indicates significant difference from control egg eclosion

Agitation Treatments. The addition of agitation or additional rinsing with disinfectants significantly increased the disinfection rate ($F = 16$; df = 9; $P < 0.0001$), but unfortunately, significantly lowered the eclosion rate (F = 9.595; df = 9; $P < 0.0001$) (Fig. 5.5). Agitation in SporGon®, dilute bleach or Lysol® for 5 min disinfected 47, 6.67 and 66.67% of the samples, respectively. Additional SporGon®, dilute bleach or Lysol® rinses disinfected 37%, 13.33 and 60% of the samples, respectively. Agitation in insect saline agitation did not affect the disinfection of the eggs, while additional insect saline rinses disinfected 3% of the samples (Fig 5.5).

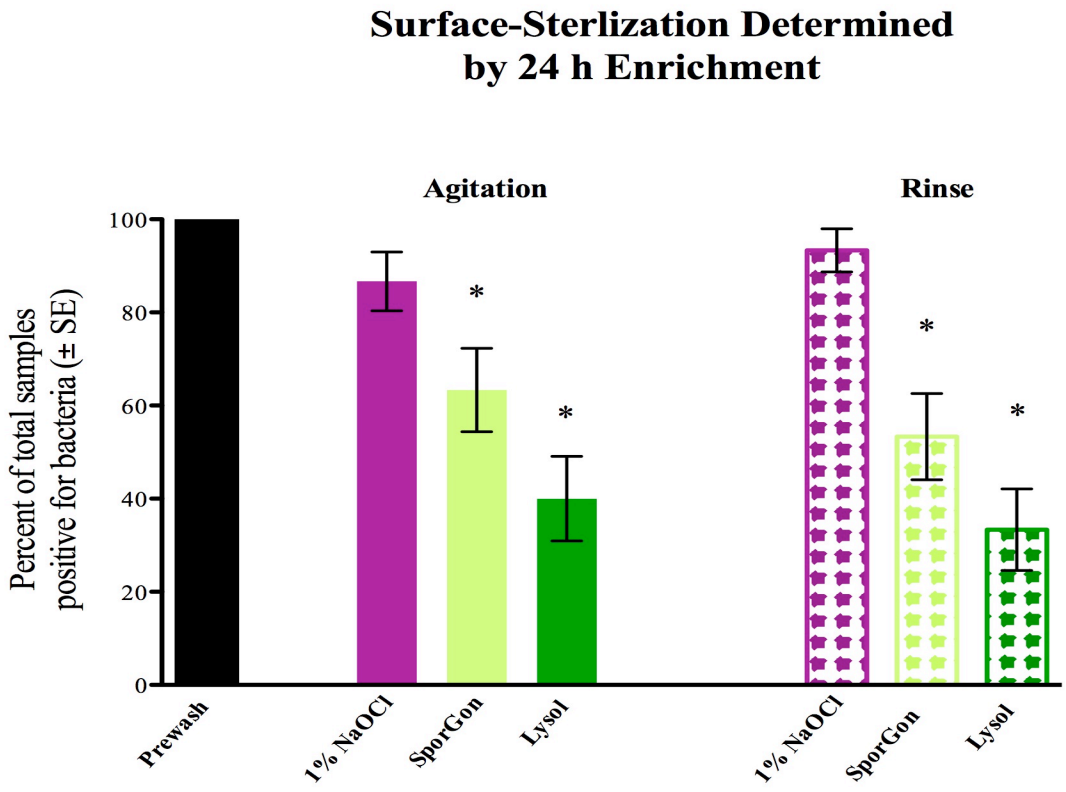


Fig 5.5. Results of surface-sterilization protocols with the addition of agitation or rinse on *Lucilia cuprina* eggs (N = 30) percent of samples positive for bacteria after surface sterilization and 24 h enrichment in TSB at 37 °C. * indicates significant difference from PRE-wash mean positive samples.

Agitation in SporGon®, dilute bleach or Lysol® lowered eclosion percentages to 57, 59 and 57%, respectively. Additional SporGon®, dilute bleach or Lysol® rinses resulted in 58, 61 and 50% eclosion, respectively. The mean eclosion for insect saline treated eggs was 83%. Agitation in insect saline resulted in 47% eclosion and additional insect saline rinses yielded 68% eclosion. All agitation and rinse treatments significantly lowered eclosion rates ($F = 9.595$; df = 9; $P < 0.0001$) (Fig. 5.6).

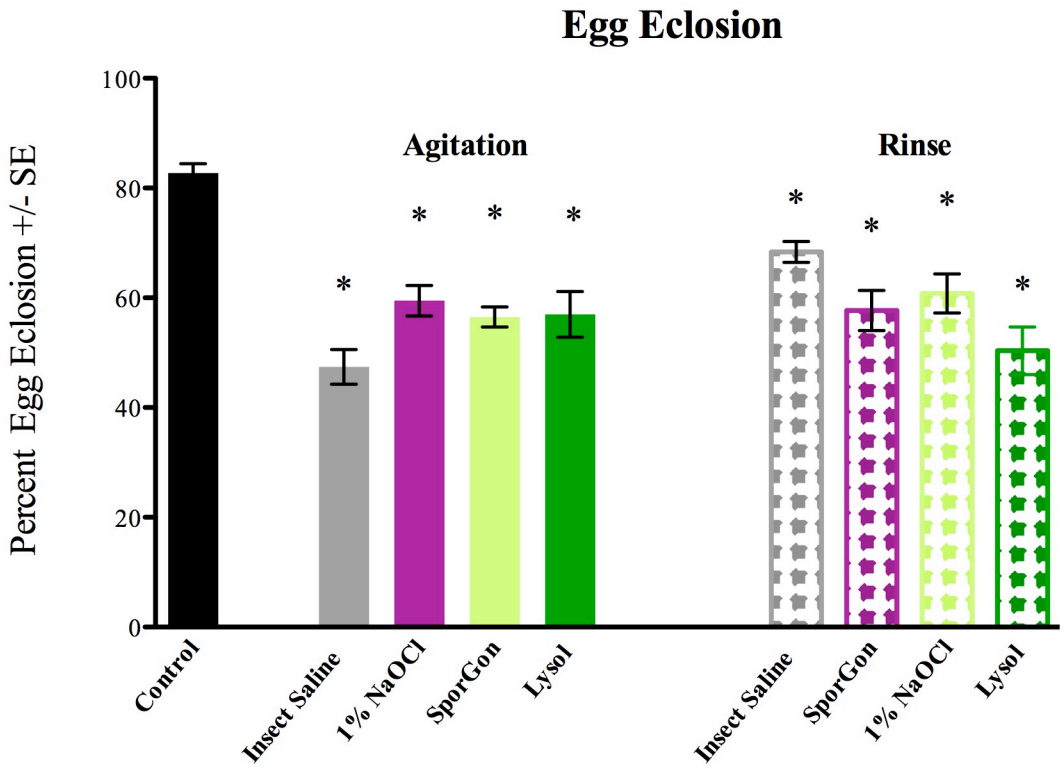


Fig 5.6. Results of surface-sterilization protocols with the addition of agitation or rinse on mean number of *Lucilia cuprina* eggs (N = 30) that successfully eclosed after treatment protocols. * indicates significant difference from control egg eclosion.

Additional Species Tests. Analysis of initial disinfection protocols and agitation treatments showed that the 10 min immersion in Lysol® yielded the highest disinfection

rates coupled with the highest mean eclosion rates. However, a survey of entomology labs revealed that H_2CO is the most commonly disinfectant used for surface sterilization of blow fly eggs (Sherman, personal communication). The efficacy of these two treatments was tested on three additional species: *Ch. rufifacies, C. macellaria,* and *L. sericata.* PRE-wash analysis showed that each species carried a significantly different bacterial load ($F = 7230000$; df = 2; $P < 0.0001$). *Chrysomya rufifacies* carried the highest mean initial bacterial load, followed by *C. macellaria,* and finally *L. sericata* (Fig. 5.7).

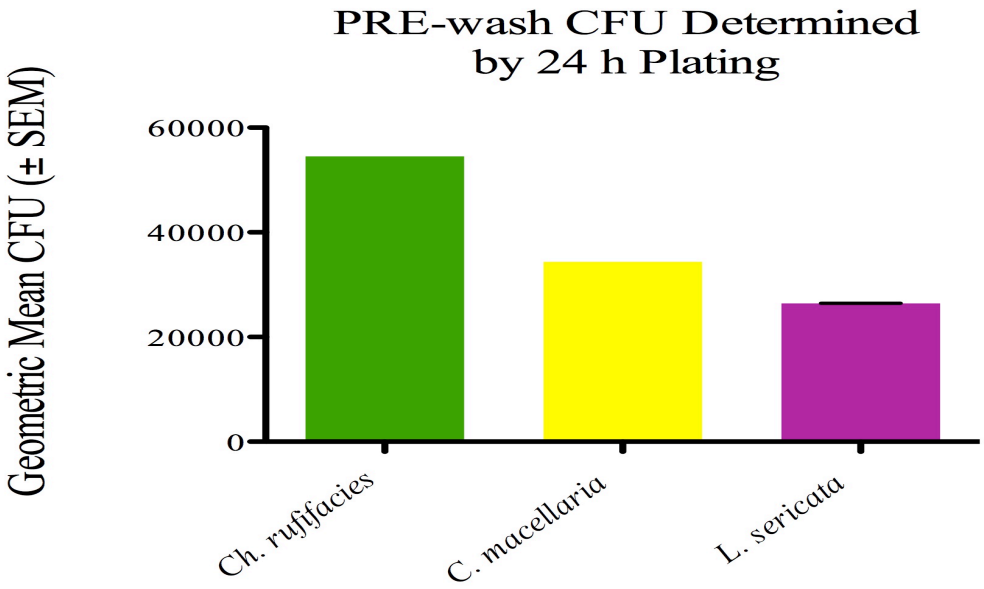


Fig 5.7. Initial bacterial load of *Ch. rufifacies, C. macellaria,* and *L. sericata* determined by 24 h culture at 37 °C on blood agar.

POST-wash enrichments showed significant differences in disinfection rates between species and treatments ($F = 7230000$; df = 8; $P < 0.0001$). Tukey post hoc showed that immersion in Lysol® for 10 minute significantly lowered percent of samples

harboring residual bacteria after 24 h enrichment for *Ch. rufifacies* ($M = 0$; SD = 0) *C. macellaria* ($M = 0$; SD = 0), and *L. sericata* ($M = 20.00$; SD = 40.68). Post hoc tests showed that H_2CO was not as effective as Lysol®, however. H_2CO significantly percent of samples harboring residual bacteria after 24 h enrichment for *C. macellaria* ($M =$ 73.33; SD = 44.98) and *L. sericata* ($M = 43.33$; SD = 50.40), but did not for *Ch. rufifacies* ($M = 83.33$; SD = 27.90) (Fig 5.8).

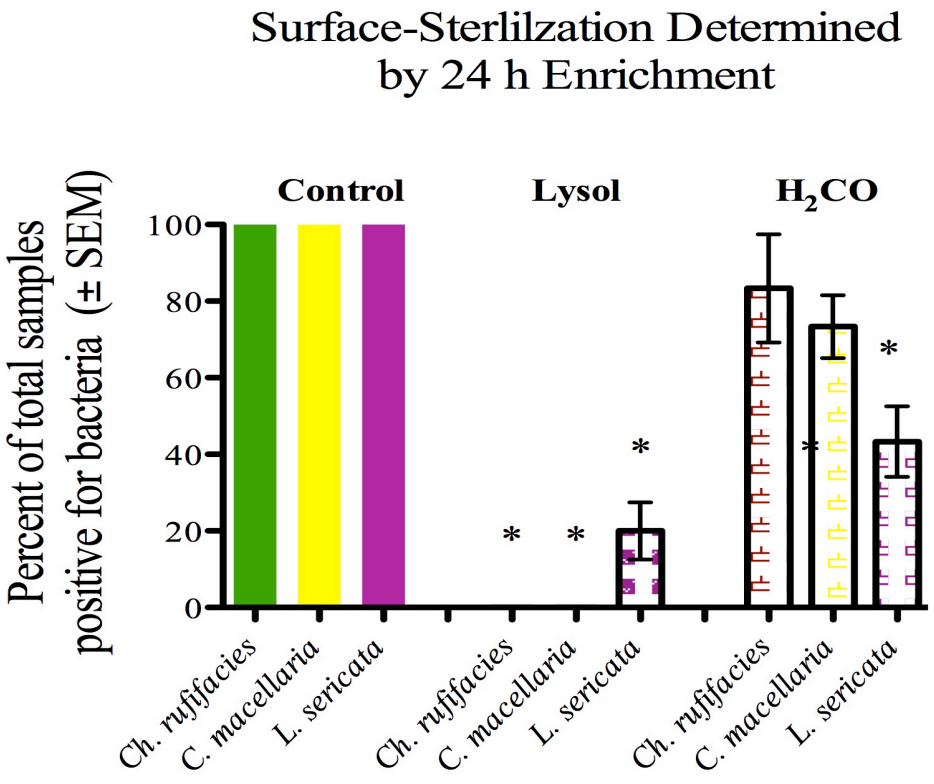


Fig 5.8 Results of surface-sterilzation protocols on *Chrysomya rufifacies, Cochliomyia macellaria,* and *Lucilia sericata.* Percent of samples positive for bacteria after surface sterilization and 24 h enrichment in TSB at 37 °C, * indicates significant difference from PRE-wash mean positive samples.

Lysol® and H_2CO treatments significantly ($P < 0.0001$) affected eclosion rate for *Ch. rufifacies* and *L. sericata,* but did not affect eclosion rates of *C. macellaria*. Mean eclosion for untreated *Ch. rufifacies* eggs was 91.22%, for *C. macellaria* eggs was

87.50%, and for *L. sericata* eggs was 70.90% (Fig. 5.9). Immersion in Lysol® for 10 minute lowered eclosion percentages of *Ch. rufifacies* by 27.65%, and *L. sericata* by 11.15%. *Cochliomyia macellaria* eclosion rates were not affected by the treatment. H_2CO treatment lowered *Ch. rufifacies* egg eclosion by 14.47%. *Lucilia sericata* and *C. macellaria* eclosion rates were unaffected by the treatment (Fig. 5.9).

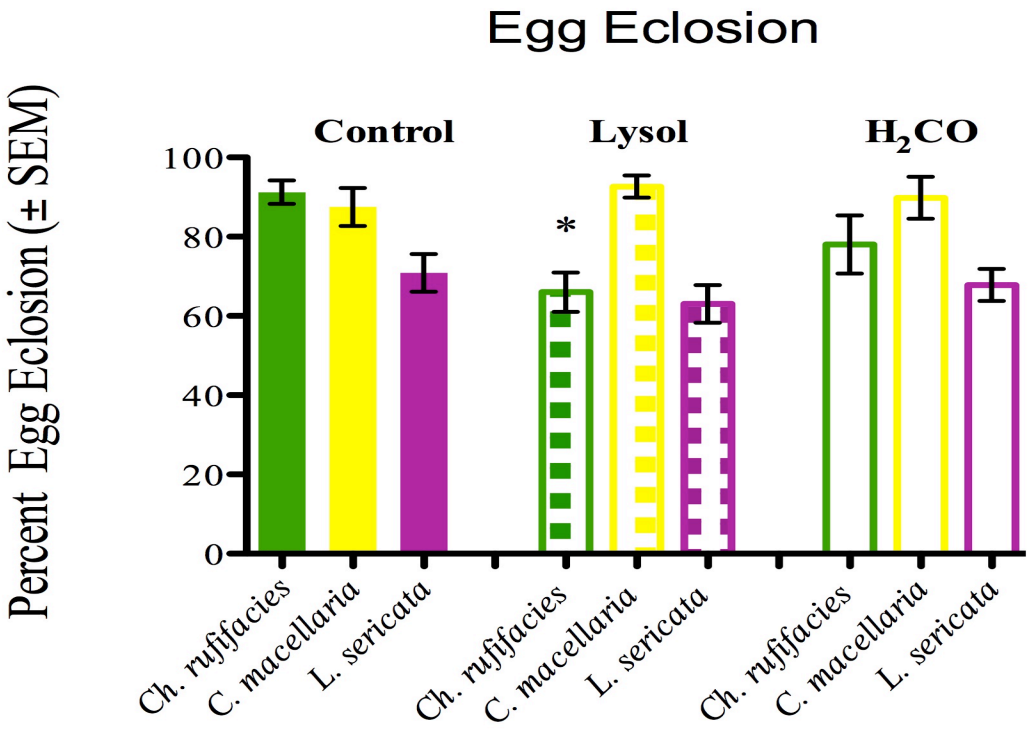


Fig 5.9.Results of surface-sterilzation protocols on *Chrysomya rufifacies, Cochliomyia macellaria,* and *Lucilia sericata:* on mean number of eggs that successfully ecosed. * indicates significant difference from control egg eclosion.

Chorion visualization with potassium permanganate revealed the effect of each treatment on the outer surface of the eggs. Both untreated and saline rinsed eggs maintained an intact chorion, as did all treatments save the bleach and H_2CO immersion.

The chorion appeared completely removed by this treatment, although the vitelline membrane appeared to be intact (Fig. 5.10).

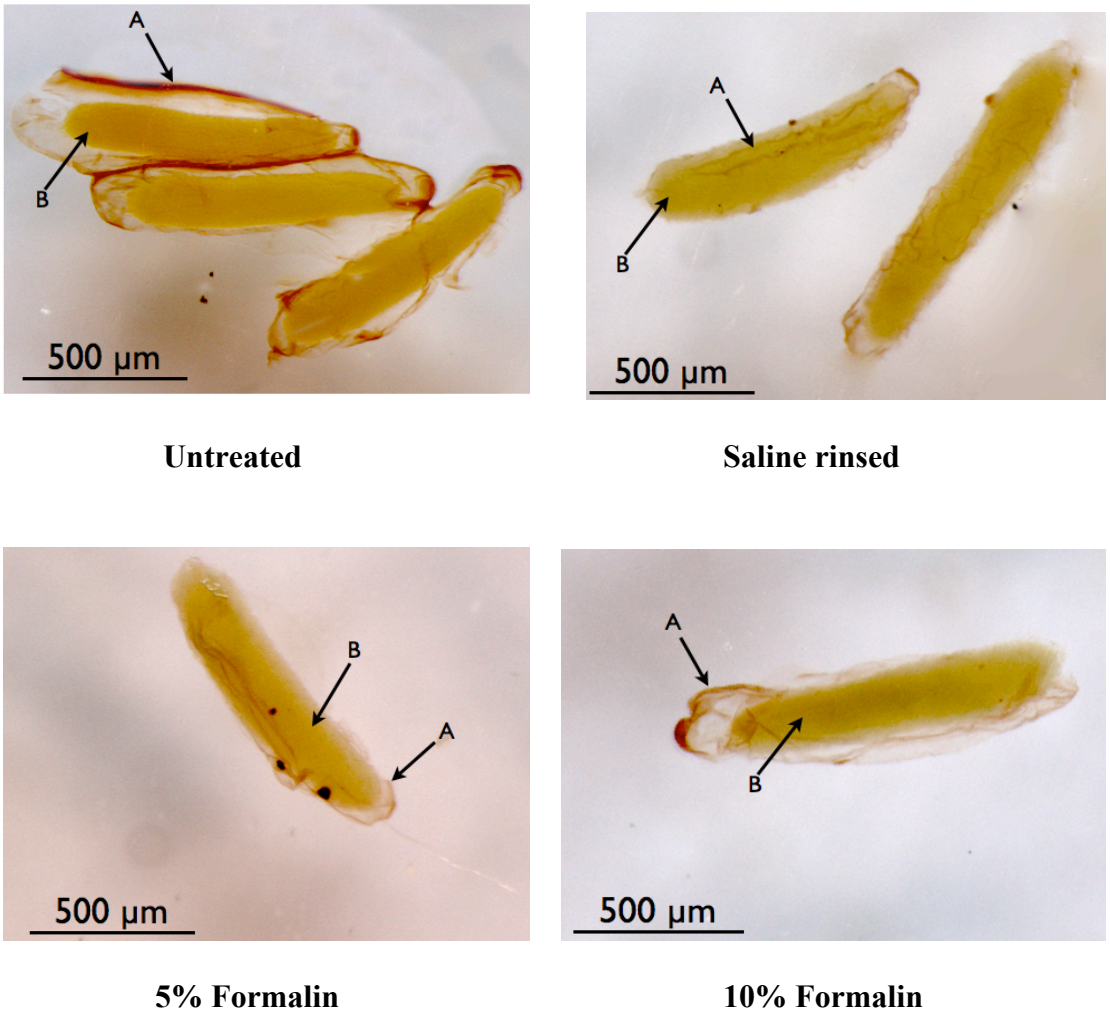
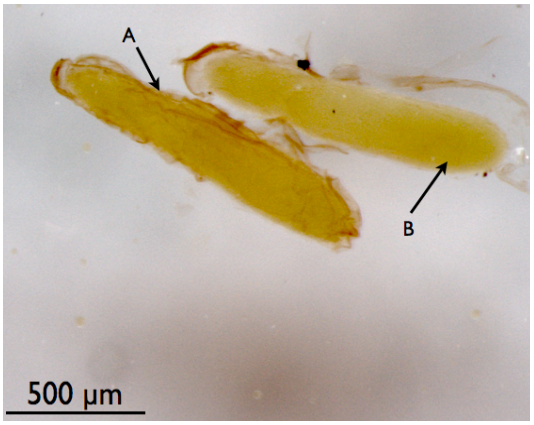
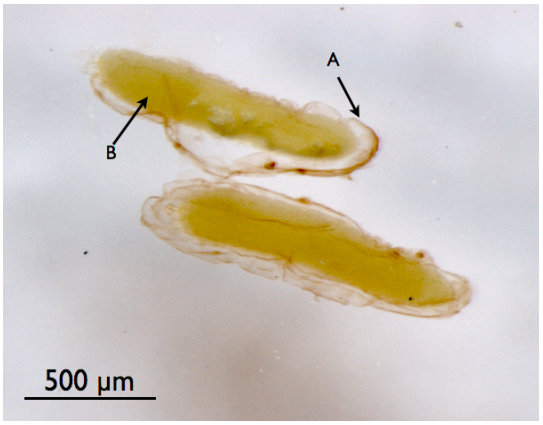


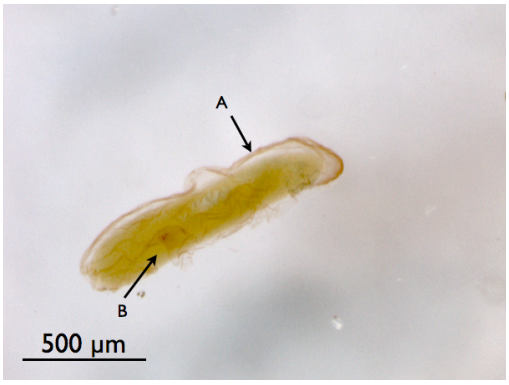
Fig 5.10 Visualization of *Lucilia cuprina* eggs before and surface-sterilization after treatments. Eggs were stained with potassium permanganate to differentiate the (A) chorion from the (B) vittellin membrane. Lack of stain on bleach treatments indicates removal of chorion by bleach.



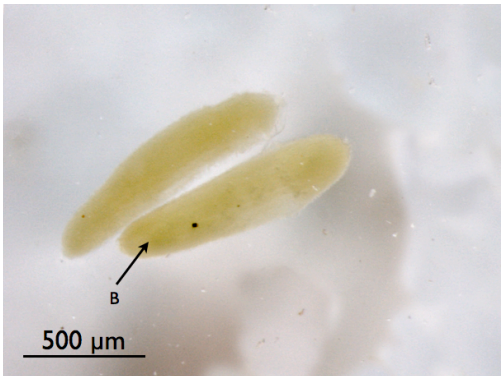
5 minute Lysol Immersion



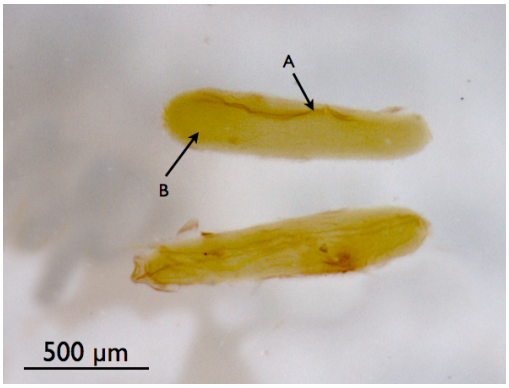
10 minute Lysol Immersion



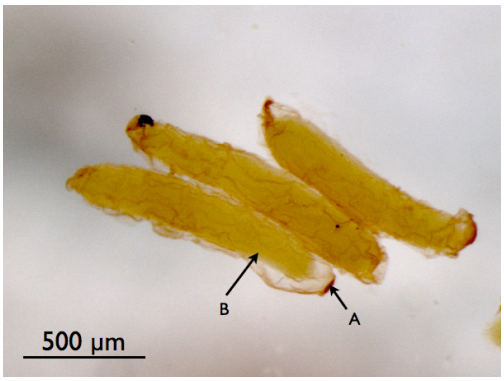
H_2CO



Bleach and H_2CO

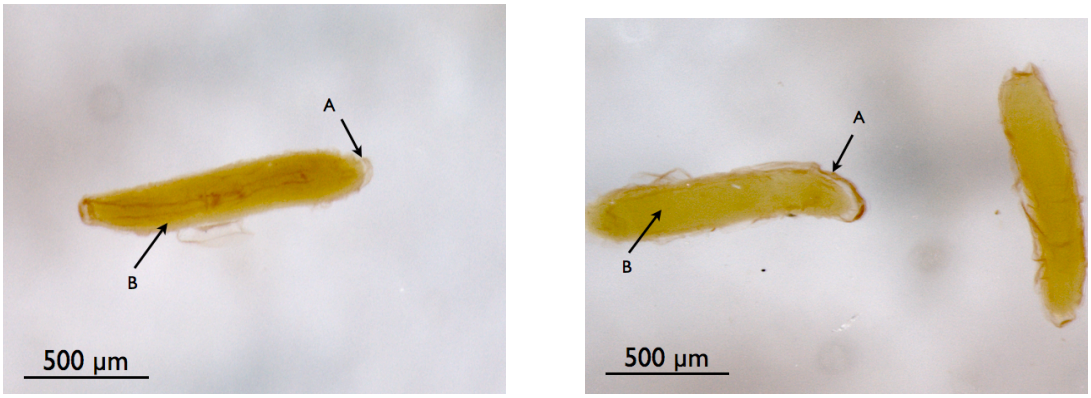


1:50 Bleach rinse



Benzalkonium Chloride

Fig 5.10 continued



70% Ethanol **95% Ethanol and SporGon**

Fig 5.10 continued

Discussion

The use of biosurgical maggots in debridement therapy is increasing partially due to the development of antimicrobial resistance in bacteria (Sherman et al. 2000, Jaklic et al. 2008). Because of the habitats in which flies breed, the use of larvae for medical proposes necessitates the surface sterilization of fly eggs prior to rearing for use as biosurgical maggots (Baer 1931, Sherman et al. 2000). Neglecting to do so risks the introduction of potentially detrimental bacteria into an already wounded individual, which may lead to secondary infection and death (Baer 1931).

Previous studies have reported on the surface sterilization of blow fly eggs (Baer 1931, Greenberg 1970, Connell 1981, Sherman and Tran 1995, Wollina 2000, Figueroa et al. 2007). However, validation of these methods on medically important species was either not presented, or the effect of the sterilization methods on hatch rates was not

investigated. In this study, the efficacy of disinfection treatments previously or currently used in the production of medical maggots to remove culturable, aerobic bacteria from the external surface of *L. cuprina* were assessed in conjunction with the subsequent hatch rates of the disinfected eggs. *Lucilia cuprina* was selected as a sentinel species due to its high sensitivity to immersion in liquid. The resulting most effective treatment was then tested on three additional medically important species, *Ch. rufifacies, C. macellaria,* and *L. sericata;* along with the most common protocol current used in MDT labs commercially producing medical maggots

The amount of bacteria carried by the eggs of the four species ranged from 3.44 x 10^4 to 12.99 x 10^4 CFU/ml. Bacterial load is subject to many factors, such as oviposition medium (Banjo et al. 2006, Costello et al. 2009, Barnes and Gennard 2011), moisture (O'Keefe and Schorsch 1954, Rejmankova et al. 2000), ambient temperature (Rejmankova et al. 2000, Zavarzin 2008), fecal-spots (Lerch 2003, Dillon and Dillon 2004), and oviposition fluids associated with egg deposition (Lam et al. 2007, Lam et al. 2009). Given the variation in bacterial load, the use of a large sample size is necessary for disinfection efficacy studies.

Currently, H_2CO in used as the primary disinfectant for blow fly eggs in laboratories producing biosurgical maggots (Sherman et al. 2000, Figueroa et al. 2007, Gericke and Pitz 2008, Jaklic et al. 2008). This study found that the efficacy of disinfection by H_2CO was significantly lower than other treatments and led to fewer eggs eclosing in most species. H_2CO treatments were most effective on *L. sericata* than other species, possibly indicating a community of bacteria inhabiting the chorion that is

more susceptible to H_2CO. Additionally, *L. sericata* eggs were not as easily killed by H_2CO as other species.

In an effort to increase the effectiveness of H_2CO disinfection treatments, immersion of the eggs in bleach prior to H_2CO immersion was performed (Mumcuoglu et al. 2001). Bleach is commonly used as a sterilization agent (Rutala 1996) and should therefore adequately disinfect bacterially contaminated surfaces. However, bleach is also known to remove the chorion from eggs (Connell 1981) and retard embryological development (Connell 1981). In this study, a pre-wash with bleach increased the disinfection efficacy of H_2CO without significantly lowering hatch rates. However, even with the addition of a pre-wash with bleach, near 100% disinfection was not reached, and the eclosion rates were significantly lower than controls, which precludes this treatment from being efficient for large-scale egg sterilization.

Since H_2CO is an inherently volatile and dangerous substance, some investigators elected to use formalin, a commercial form of dilute H_2CO mixed with a stabilizer (Simmons 1934). While this formulation of H_2CO allows for long-term storage of the disinfecting agent, and is considered a safer alternative, it did not adequately disinfect the egg surface. Evaluation by bacterial enrichment post 5 and 10% formalin treatment showed that nearly 96.67 and 56.67%, respectively, of the treated eggs harbored some bacteria; while only 36.67% of H_2CO treated eggs harbored bacteria. This lowered disinfection efficacy may have been due to the smaller amount of H_2CO present in formalin formulations.

In an effort to reduce the chorion removal due to bleach treatments, Teich (1986) diluted the bleach 1:50 and washed the solution over the eggs, but did not quantify the surface disinfection rates (Teich 1986). Evaluation of results in this study showed that continuous rinsing coupled with the disinfection of the NaOCl resulted in moderate disinfection, and a very high eclosion rate. This may be due to the limited exposure the chorion had to the bleach. Since the eggs were bathed in a dilute bleach solution, the bleach may not have had time to remove the chorion as seen in full-strength bleach soaks. This would account for the high eclosion rate. However, dilute bleach did not disinfect the surface of the eggs adequately, lowering mean CFU at the same rate as formalin and H_2CO.

ADBAC is used as a disinfecting agent for water baths in laboratories (Rutala 1996), and should therefore have the capacity to disinfect surfaces. This treatment significantly lowered initial bacterial load on egg surfaces, yet 24 h enrichment for bacteria showed 70% of the eggs still harboring residual bacteria on the egg surface (Fig. 5.2). This indicates that the majority of culturable bacteria are initially killed, but that the surface of the eggs is not completely sterilized.

EtOH is used in many situations as a sterilization mechanism (Rutala 1996), and has been used to surface disinfect blow fly eggs prior to MDT (Brookes 1961). In our study, 70% EtOH was one of the least efficient disinfecting agents tested, but it had low toxicity to egg eclosion. Despite significantly lowering bacterial load on egg surfaces below control levels, it left significantly more bacteria on the eggs than all other treatments, except 5% formalin, which demonstrated a similar lack of disinfection

efficacy. This may have been due to the limited amount of time the ethanol spent in contact with bacterial contaminants. A longer ethanol soak may increase the efficacy of this treatment, but may also affect egg mortality.

The addition of SporGon® to the ETOH rinse was based on surface sterilization of beetles (Crippen 2006). SporGon® is a commercial formula designed to kill spore-forming bacteria, and surface sterilize lab equipment. This treatment was effective for the surface sterilization of beetles, leading to near 100% sterilization of contaminating bacteria (Crippen 2006). This treatment did not significantly lower the eclosion rate of treated eggs, but it also did not completely disinfect the egg surfacing, leaving substantial residual bacteria behind that emerged after 24 h incubation. SporGon® does not include a surfactant in its formulation; therefore it may be unable to access the entire surface of a clumped clutch of eggs. This hypothesis led to the addition of agitation to the treatment protocol to separate the agglutinated eggs. Agitation increased the disinfection efficacy of SporGon® by 20%, but unfortunately it also decreased the eclosion rate by 15%. Agitation alone was found to decrease eclosion rate by 21%. So SporGon® may be ineffectual for egg sterilization due to a lack in its formulation of compounds to deagglutinate the eggs, resulting in the inability to access bacteria insulated within egg clutches. However, we found that agitation of the eggs during disinfection is also not recommended due to their apparent sensitivity to this type of manipulation.

Lysol® immersions resulted in the highest disinfection efficacy coupled with the best egg eclosion rates. Five-minute immersion in Lysol® resulted in an 80% reduction

of bacterial contaminants, with only 20% of the samples showing residual bacteria after 24 h enrichment, and an eclosion rate not significantly different from untreated eggs. Lysol® immersion for 10 min resulted in 97% disinfection egg samples; only one of the 30 samples yielded residual bacteria after 24 h incubation. This treatment's eclosion rates were also not significantly different from controls. This treatment yielded the highest rate of surface disinfection of the eggs, and the accompanying high eclosion rate makes this treatment the most effective of those tested.

The 10 minute immersion in Lysol® was compared to the commonly used 5 min immersion in H_2CO on three additional species. H_2CO did not significantly reduce surface bacteria on *Ch. rufifacies* eggs, but lowered the bacteria on *C. macellaria* eggs by 20%, and on *L. sericata* eggs by 60%. The 10 minute immersion in Lysol® resulted in 100% disinfection of both *Ch. rufifacies* and *C. macellaria* eggs, and a reduction of bacteria on *L. sericata* of 80%. Egg eclosion remained high for all three species under both treatments, although *Ch. rufifacies* eclosion was reduced by 30% after the Lysol® immersion. The combination of high disinfection rates along with high eclosion rates makes a 10-min Lysol® immersion the most efficacious surface disinfection for four species of biosurgical maggots.

During oviposition, eggs are laid in groups or clutches on the oviposition medium. The clutches are covered with a layer of glycoprotein that may prevent dehydration, and adheres the egg clutch to the substratum (Peterson 1991). This glycoprotein layer may be responsible for the adhesion of bacteria to the egg surface. Inadequate deagglutination of the egg clutches prior to disinfection results in poor

surface sterilization (Sherman 1996). The failure of several of our treatments may be due to the inadequate deagglutination of egg clutches, and the subsequent protection of bacteria within glycoprotein layers. Historically, egg clutches were deagglutinated using NaOCl (Weil et al. 1933, Connell 1981, Sherman 1996), which resulted in chorion removal and decreased egg eclosion (Connell 1981). The physical deagglutination used in this experiment attempted to maximize both egg disinfection and egg eclosion. It may be that not chemically removing the deposited glycoprotein from the egg clutches prevented some of the disinfectants from reaching trapped bacteria, thereby allowing for continued contamination. Lysol® is formulated with a commercial surfactant, which breaks down the glycoprotein without damaging underlying tissues. This formulation possibly enabled the disinfectant to reach the bacterial contaminants while still allowing for adequate egg eclosion after surface sterilization.

The cultivation of medical maggots can be a time intensive process. Although it is possible that eggs harbor bacterial organisms internally (Sherman et al. 2000), when preparing larvae for medicinal uses, surface disinfection of the eggs is crucial to a positive therapeutic outcome. Insufficiently surface sterilized eggs can contaminate wounds and lead to secondary infection (Weil et al. 1933). Several previous studies used protocols that purportedly disinfected the surface of eggs (Glaser 1938, Horn et al. 1976, Sherman 1996, Mohd Masari et al. 2005); however, data validating the efficacy of the described techniques, and the impact of such techniques on egg eclosion was not sufficiently presented. This study assessed the efficacy of previously described and newly developed methods to disinfect the external surface of Calliphoridae eggs. The

goal of this study was to develop a protocol that maximized surface disinfection of the eggs and minimized toxicity resulting in reduced eclosion. It is important to commercial endeavors to have a protocol producing a high yield of useable biosurgical larvae. A 10 min immersion in Lysol® removed culturable, aerobic bacteria from 97% of the external surface of three species of Calliphoridae eggs, while allowing for high rates of egg eclosion.

CHAPTER VI

GENERAL CONCLUSION

These experiments were designed to investigate the mechanisms involved with colonization of an ephemeral resource by two forensically important flies, *Chryosmya rufifacies* (Macquart) and *Cochliomyia macellaria* (Fabricius) (Diptera: Calliphoridae). Given the biology and ecology of these species, elucidation of these mechanisms included analysis of arrival time effects on each species; effects of decomposition state of the resource; and effects of prior conspecific and heterospecific colonization of the resource on oviposition behavior. The results of these experiments have yielded some interesting facts about the behavior of these two species, and revealed a possible inter-kingdom signaling mechanism that deserves further investigation.

This research demonstrated a priority effect between *Ch. rufifacies* and *C. macellaria*, and implied that the predator-prey relationship between the two species may be more important than competition for food. The development of *C. macellaria* was impacted by the variation in arrival on a resource between itself and *Ch. rufifacies* arrival on a resource. These results demonstrate that arrival sequence significantly affects the fitness of both *Ch. rufifacies* and *C. macellaria*. Early colonization may allow the competitively weaker *C. macellaria* to persist in a community, while delaying colonization after *C. macellaria* appears to benefit *Ch. rufifacies*. Selection for such traits may explain how *C. macellaria* is able to persist in the environment despite intense predation pressure, while *Ch. rufifacies* is able to persist in the environment despite the risky behavior of secondary colonization.

The observed priority effect between *Ch. rufifacies* and *C. macellaria* indicated the use of ovipositional cues to mediate colonization of a resource. Resource age was a natural next step in the investigation, as necrophagous flies tend to colonize decomposing resources over time, and may therefore require the resource to be in a particular state of decay. The positive response of *C. macellaria* to both fresh and putrefied resource and the negative response of *Ch. rufifacies* to putrefied resource indicated that resource state alone was not enough to induce oviposition behaviors. These results led to questions about what input is necessary for oviposition on a resource, and how those inputs change over time.

Since resource age did not completely explain the observed patterns of colonization of *C. macellaria* and *Ch. rufifacies*, this work focused on the group oviposition aspect of colonization in an attempt to discover why possible competitors would oviposit in large groups. the work of Lam et al. (2007) indicated that attractancy to eggs changes as the eggs age in muscids (Diptera: Muscidae), and it was probable that calliphorids followed the same patterns. Therefore, egg age was used as a basis for comparing changing conspecific and heterospecific attractancy for *C. macellaria* and *Ch. rufifacies.* The results from initial egg attractancy experiments drove the remainder of the research.

Attractancy to both conspecific and heterospecific eggs changed based on egg age and physiological state of adults. *Chrysomya rufifacies* was attracted to both conspecific and heterospecific eggs when they were fresh, but switched to only being attracted to heterospecific eggs as they aged over 3 h. Only non-gravid females showed any

attractancy to the eggs as they approached eclosion. *Cochliomyia macellaria* were attracted to conspecific eggs and repelled from heterospecific eggs until the eggs neared eclosion, when only the males showed any preference. These data indicate that there is something about the eggs themselves that allows adults to judge the age of the egg clutch and adjust behavior to maximize offspring fitness. Since *Ch. rufifacies* is looking for prey items, it is logical that the ovipositing adults are attracted to prey eggs until those eggs reach a certain developmental stage. After that developmental stage, the *Ch. rufifacies* larvae run the risk of missing the predatory window and would have to revert to necrophagous feeding or cannibalism. *Cochliomyia macellaria*, on the other hand, seem to avoid the predator species, indicating that selection has occurred in the few years since *Ch. rufifacies* was introduced to this country. The attraction to conspecific eggs by *C. macellaria* also indicates a selection for group oviposition, which implies that offspring will be more fit if feeding in a mass. The lack of attraction as the eggs age, however, indicates that *C. macellaria* adults may be taking steps to avoid intraspecific competition, and give the larvae maximum advantage by allowing them to compete only with similarly aged larvae. These larvae would seemingly feed at the same rate, while older larvae would have the advantage of consuming more resource more quickly.

Based on these results, egg physiology itself, or shifts in bacterial communities found on the surface of the eggs might be the mechanisms governing fly attraction. *Chrysomya rufifacies* showed attraction to surface sterilized eggs, implying the use of carbon dioxide as an attractant. This result lends credence to the idea that *Ch. rufifacies* may be a primary predator and a secondary necrophage, else the volatiles governing

attraction would be more associated with decomposing tissue as opposed to living organisms. Little attraction was found to surface sterilized eggs in *C. macellaria*, which indicates that bacterial attraction may hold the key to oviposition behaviors.

Attractancy to microbes in both species varied. High throughput sequencing of egg-associated bacteria revealed that *C. macellaria* eggs carry 39 genera, while *Ch. rufifacies* carry 31 genera. The bacterial community associated with the eggs changes over time and may affect adult behavior. *Chrysomya rufifacies* show varying levels of attraction to bacteria associated both with conspecific and heterospecific eggs, and this attraction coupled with the attraction to physiological processes of the surface-sterilized eggs goes a long way in explaining the attractancy patterns observed by this species. *Cochliomyia macellaria*, however, showed no clear patterns of attraction to microbes. This indicates the need for greater analysis of the volatiles produced by individual bacterial species, and their interactions on the egg surfaces. This information, coupled with information about olfactory cues emitted from potential oviposition sites should help elucidate the attraction of *C. macellaria* to oviposit.

This research also refined the methods used to analyze Calliphoridae response to odors in a dual choice olfactometer, and validated methods of surface sterilization of blow fly eggs. Residence time was found to be a more powerful technique when coupled with Monte Carlo simulation in analyzing the behavior of adult flies in the olfactometer. This technique allowed for significant responses where trends were initially observed.. It also took into account the physiological state of the females, as ovarian status and protein meals are known to affect behaviors in regards to resource location and

utilization. The data gathered during the olfactometer experiments allowed for refinement of olfactometer technique, and generated questions regarding other possible physiological factors that may affect response to odors.

Surface sterilization of eggs is an often-used lab technique that has seldom been validated. This work looked the most common methods of blow fly disinfection, and validated both the efficacy of the treatment protocols and the effect of the treatments on the egg chorion and eclosion rate. Lysol® was found to the most effective surface-sterilization treatment, although its efficacy depended upon the species of egg being disinfected. This indicates a variation among species in the bacterial contaminants and perhaps the substances deposited by ovipositing females.

The mechanisms that govern the attraction to an oviposition site by calliphorid flies need to be understood completely. This knowledge will lead to a greater understanding of colonization mechanisms, which, in turn, may be applied to such diverse applications as forensic entomology and livestock pest management. Since calliphorids are important in both industries, the knowledge of oviposition preference could also lead to a better time of colonization estimation in cases of human death, mechanisms for control of myiasis producing flies in livestock, and the development of more efficient traps for monitoring fly populations.

Given the results of my research, future experiments should endeavor to explore both the ecology and behavior of *Ch. rufifacies* and *C. macellaria* as competitors and possible predators and prey on a resource, along with the effect of bacteria in governing the arrival and duration of these two species. Since *Ch. rufifacies* is significantly more fit

in the presence of *C. macellaria,* the effect on the larval duration of each species should be investigated, as this has immediate practical importance in forensic entomology. Currently, life-history data sets available are for single species. No studies have examined their development when together. The fitness effects of intraspecific competition should also be analyzed to determine how these selective pressures have shaped the colonization patterns and interactions of these two species. An analysis of effects of carbon dioxide on the attractancy of both species should also be looked at, to determine if respiration has any bearing on colonization patterns for either fly. The bacterial species found on the eggs should be compared with those normally found on decomposing remains to determine if any similarities or differences are present, and perhaps elucidate the reason for the presence of these species on fly eggs. The origin of the bacteria may also shed light on how these species interact with their environment, and how bacteria interact with the flies. Further investigation of surface-sterilization procedures is also necessary to fully understand what products effectively disinfect insect eggs, and what causes the variation among the treatments. Knowledge of this variation may allow for the more effective control of medically important species and the use of those species in a medically important manner. Finally, the change in bacterial communities on the fly eggs may open a novel avenue of egg age estimation, a subject important in forensics.

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APPENDIX A

ATTRACTION OF ADULT *C. MACELLARIA* AND *CH. RUFIFACIES* TO CONSPECIFIC AND HETEROSPECIFC EGGS, FIRST CHOICE DUAL CHOICE OLFACTOMETER DATA

Description of data

These data represent the first choice responses of adult *C. macellaria* and *Ch. rufifacies* that were collected concurrently with the residence time data (Chapter IV), and analyzed with Chi Square. These data were not included with the data set for Chapter IV as the residence time data was deemed more informative.

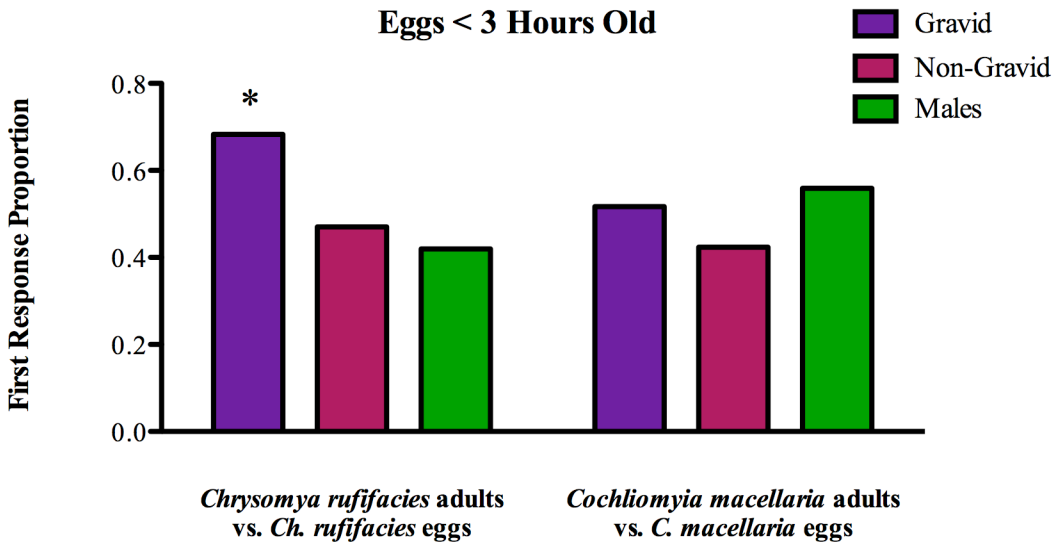

Fig A1. Proportion of adults choosing conspecific eggs as initial choice in the dual choice olfactomer.* indicates significant ($P \leq 0.05$) differences.

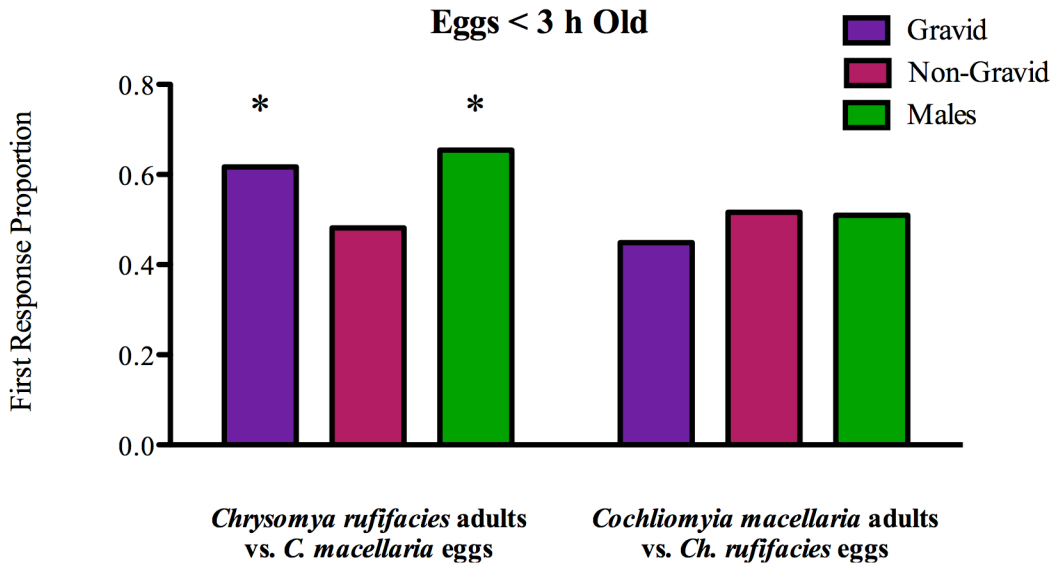

Fig A2. Proportion of adults choosing heterospecific eggs as initial choice in the dual choice olfactomer. * indicates significant ($P \leq 0.05$) differences.

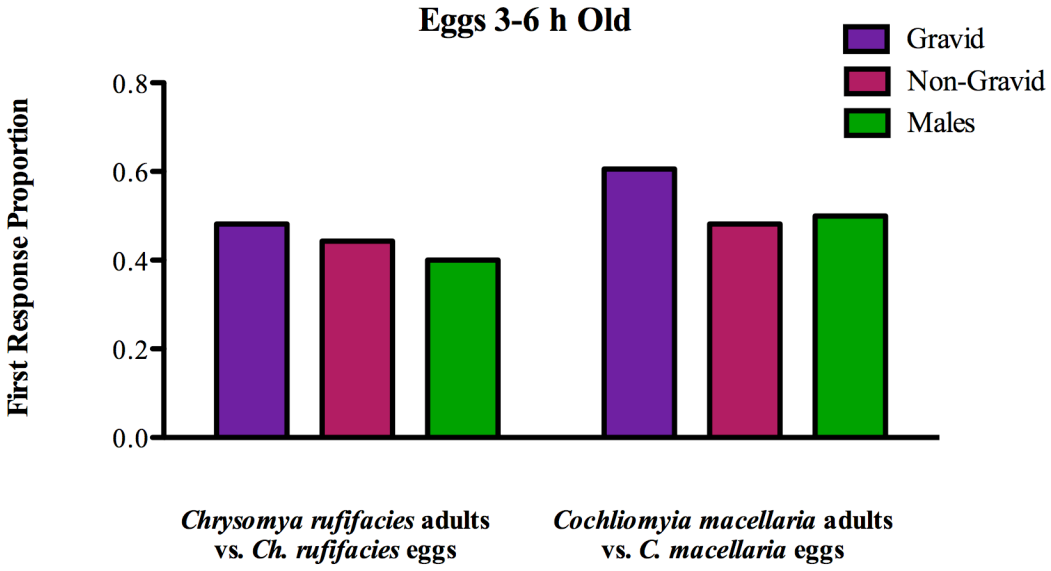

Fig A3. Proportion of adults choosing conspecific eggs as initial choice in the dual choice olfactomer. * indicates significant ($P \leq 0.05$) differences.

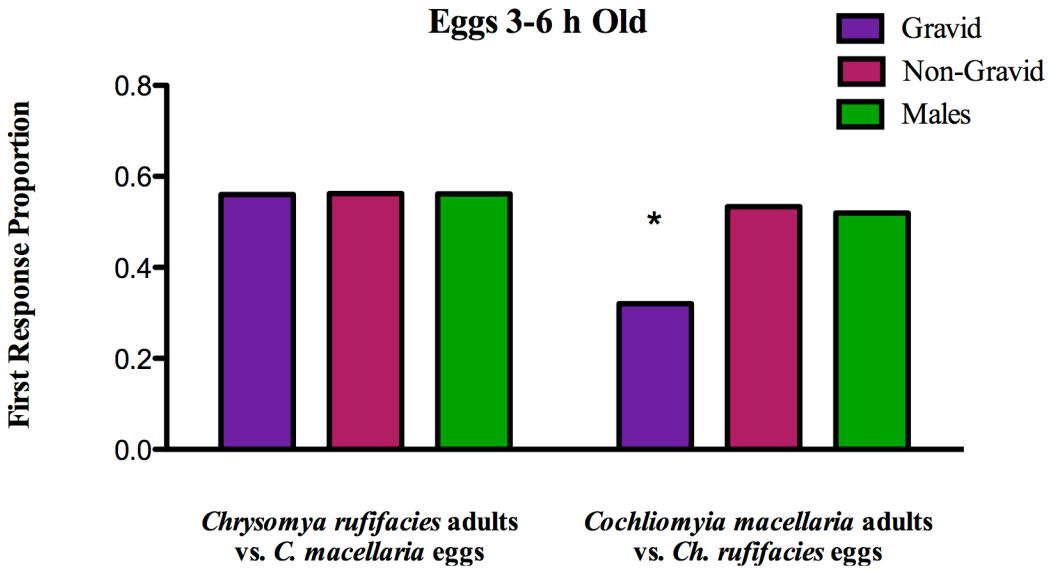

Fig A4. Proportion of adults choosing heterospecific eggs as initial choice in the dual choice olfactomer. * indicates significant ($P \leq 0.05$) differences.

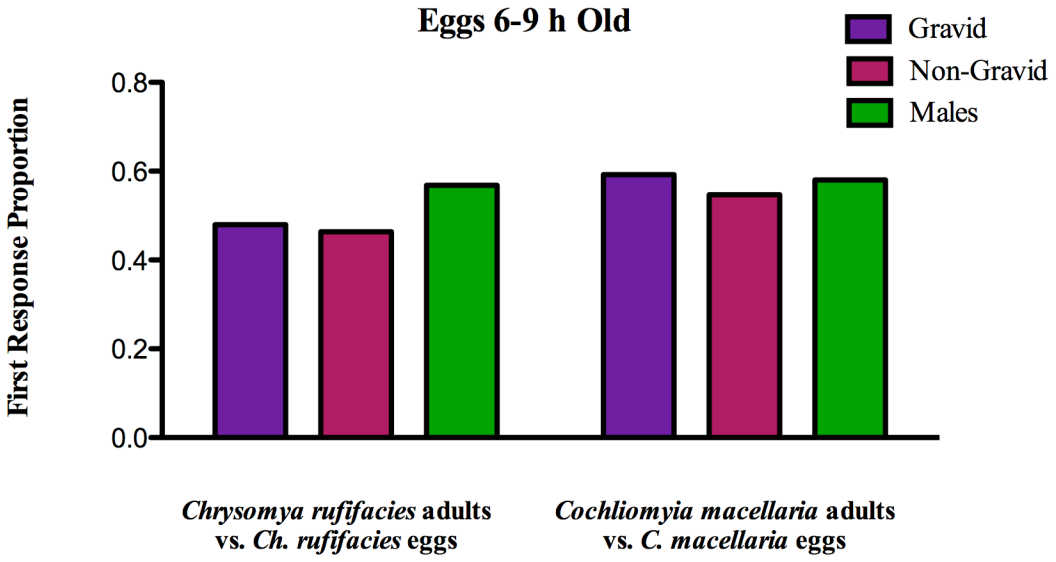

Fig A5. Proportion of adults choosing conspecific eggs as initial choice in the dual choice olfactomer. * indicates significant ($P \leq 0.05$) differences.

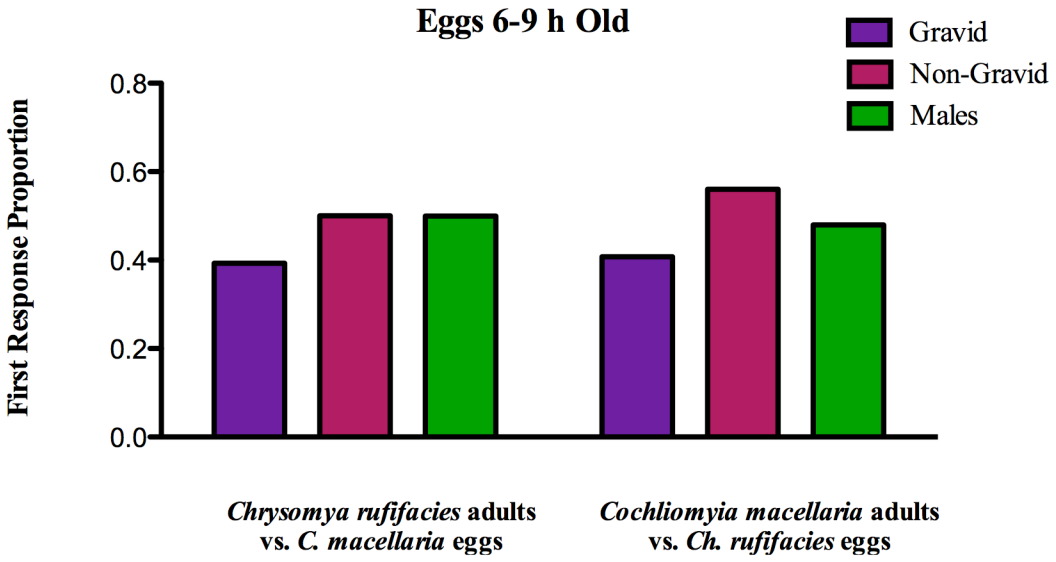

Fig A6. Proportion of adults choosing heterospecific eggs as initial choice in the dual choice olfactomer. * indicates significant ($P \leq 0.05$) differences.

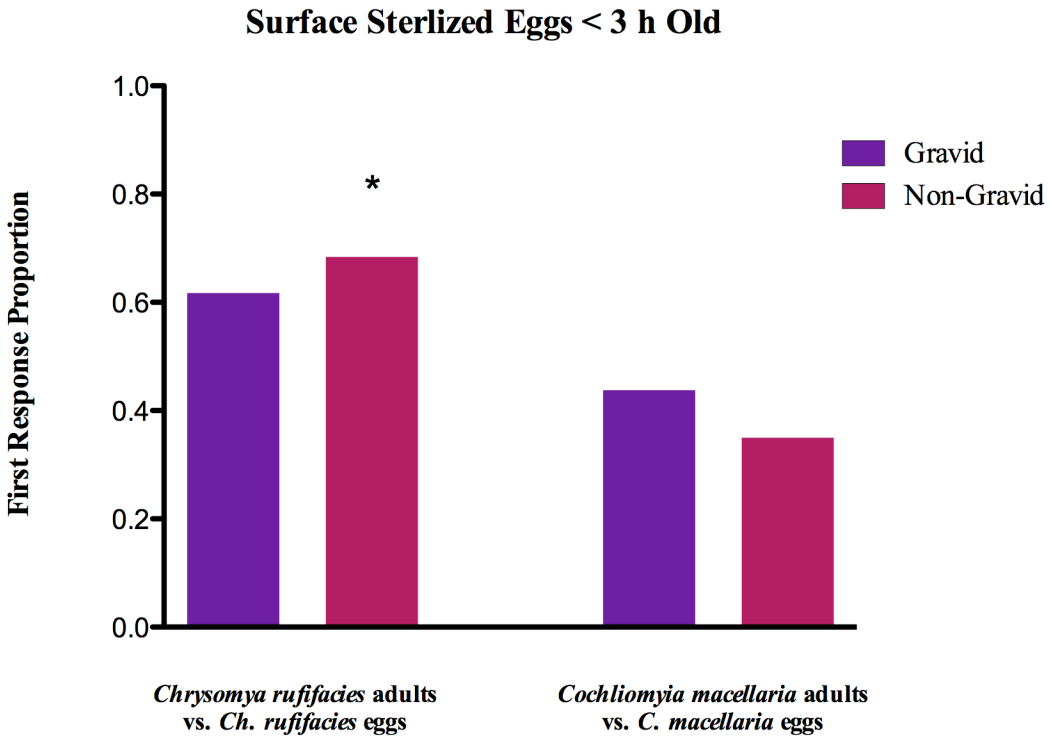

Fig A7. Proportion of adults choosing conspecific surface-sterilized eggs as initial choice in the dual choice olfactomer.
* indicates significant ($P \leq 0.05$) differences.

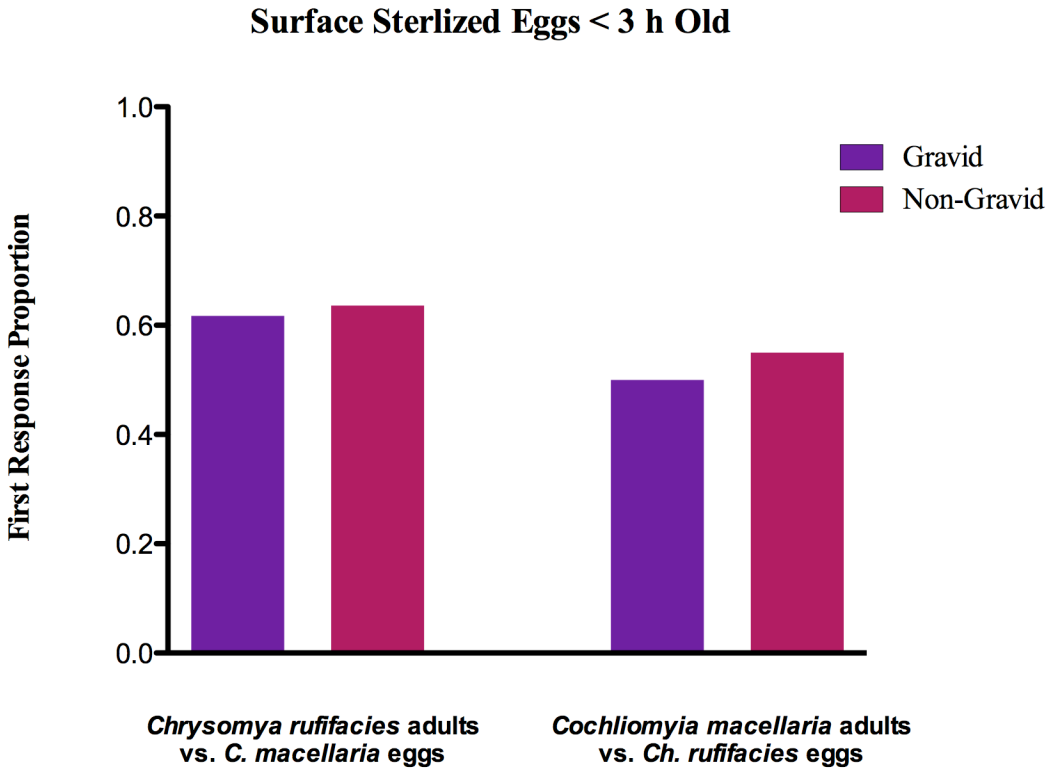

Fig A8. Proportion of adults choosing heterospecific surface-sterilized eggs as initial choice in the dual choice olfactomer
* indicates significant ($P \leq 0.05$) differences.

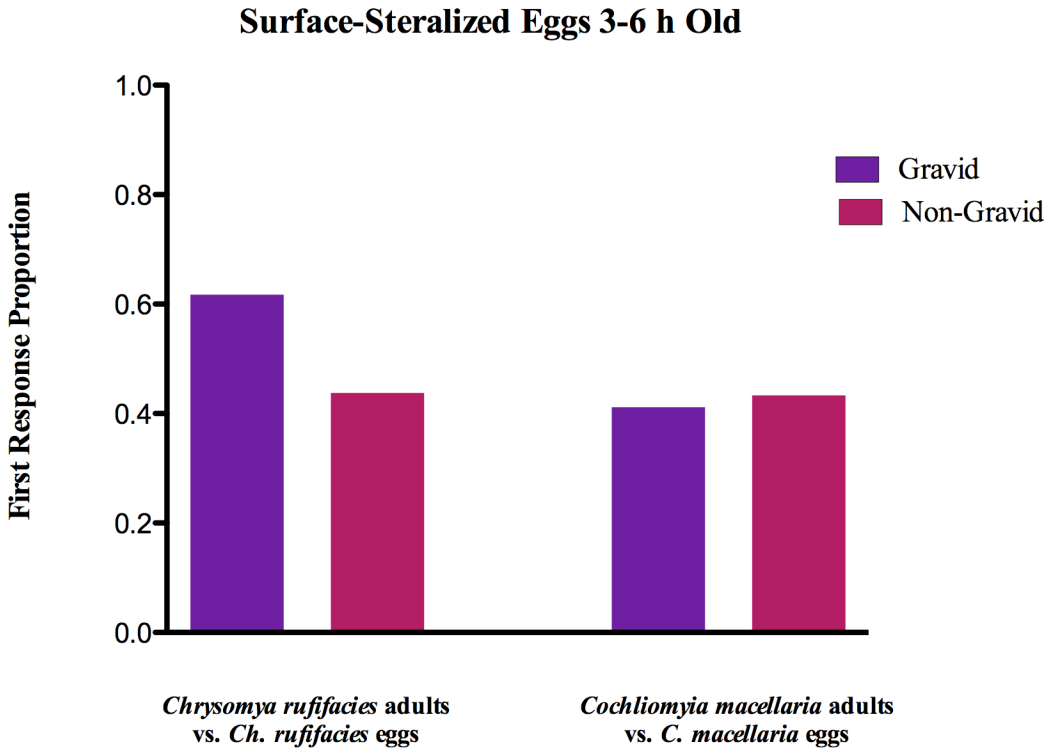

Fig A9. Proportion of adults choosing conspecific surface-sterilized eggs as initial choice in the dual choice olfactomer. * indicates significant ($P \leq 0.05$) differences.

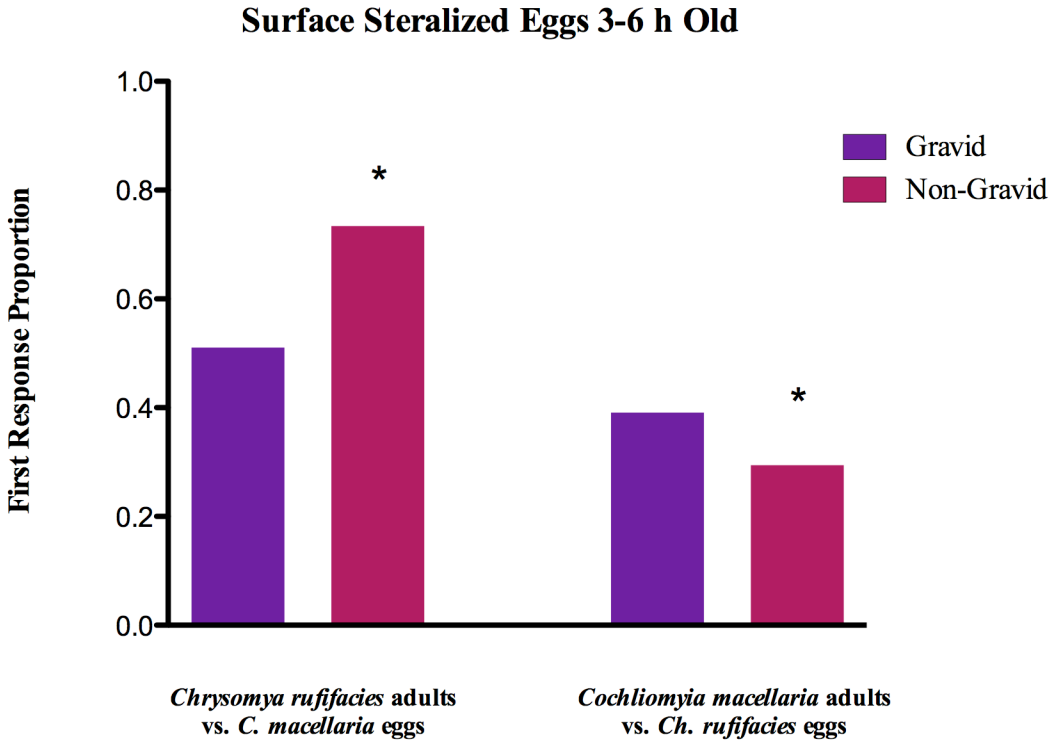

Fig A10. Proportion of adults choosing heterospecific surface-sterilized eggs as initial choice in the dual choice olfactomer. * indicates significant ($P \leq 0.05$) differences.

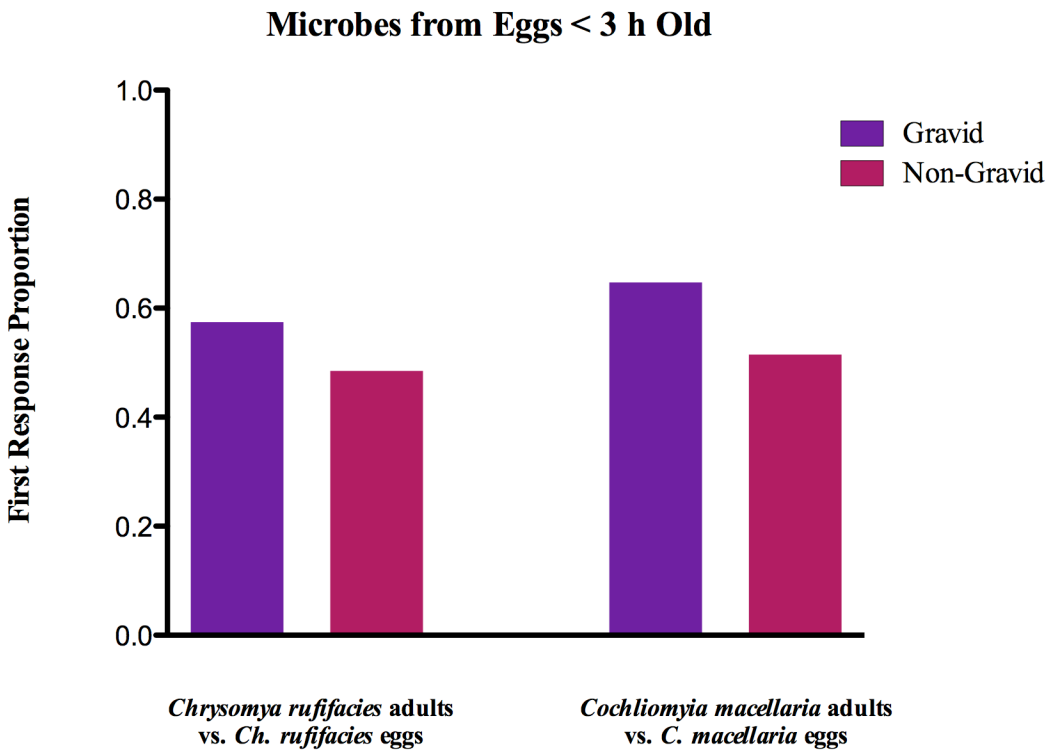

Fig A11. Proportion of adults choosing conspecific egg assoicated microbes as initial choice in the dual choice olfactomer. * indicates significant ($P \leq 0.05$) differences.

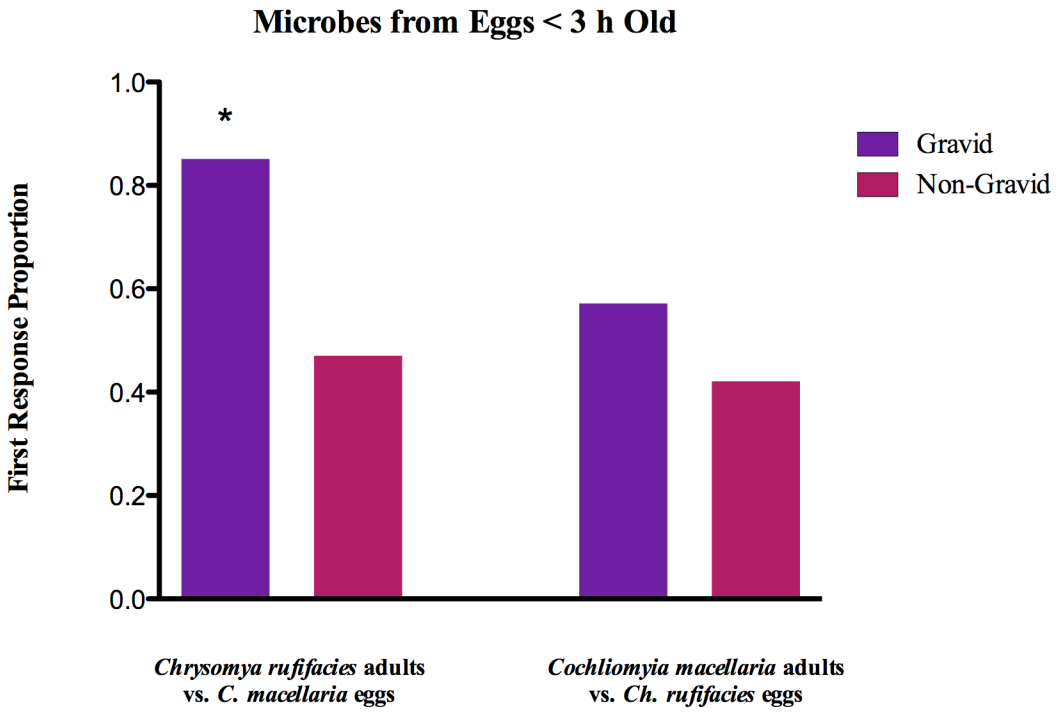

Fig A12. Proportion of adults choosing heterospecific egg assoicated microbes as initial choice in the dual choice olfactomer.
* indicates significant ($P \leq 0.05$) differences.

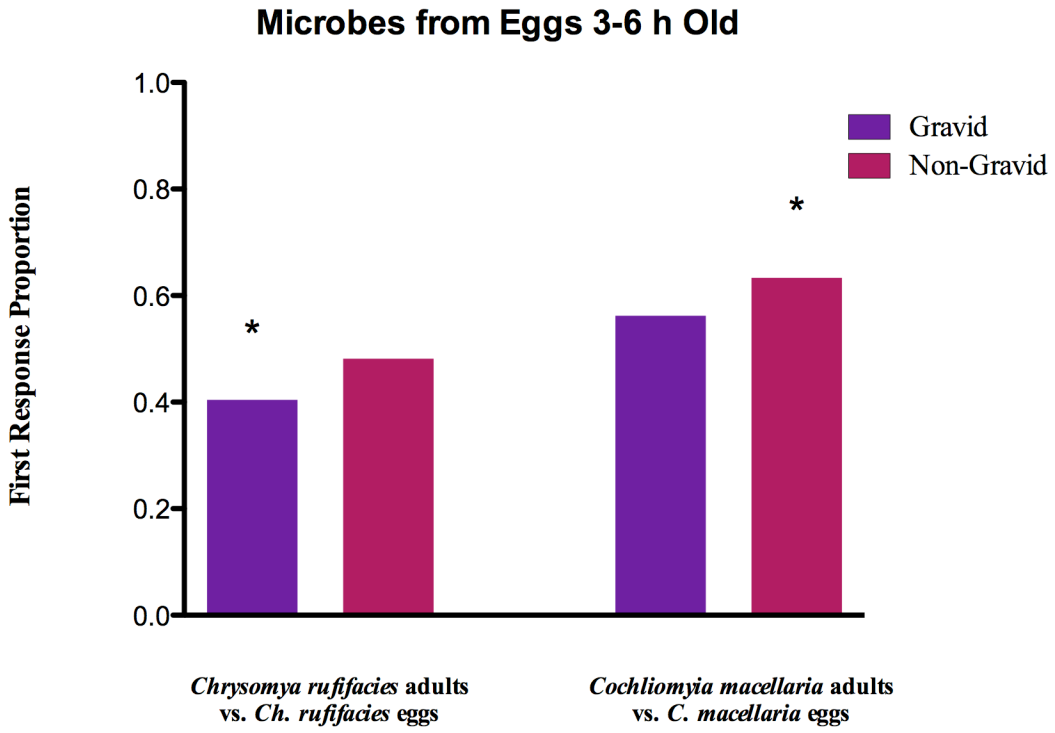

Fig A13. Proportion of adults choosing conspecific egg assoicated microbes as initial choice in the dual choice olfactomer. * indicates significant ($P \leq 0.05$) differences.

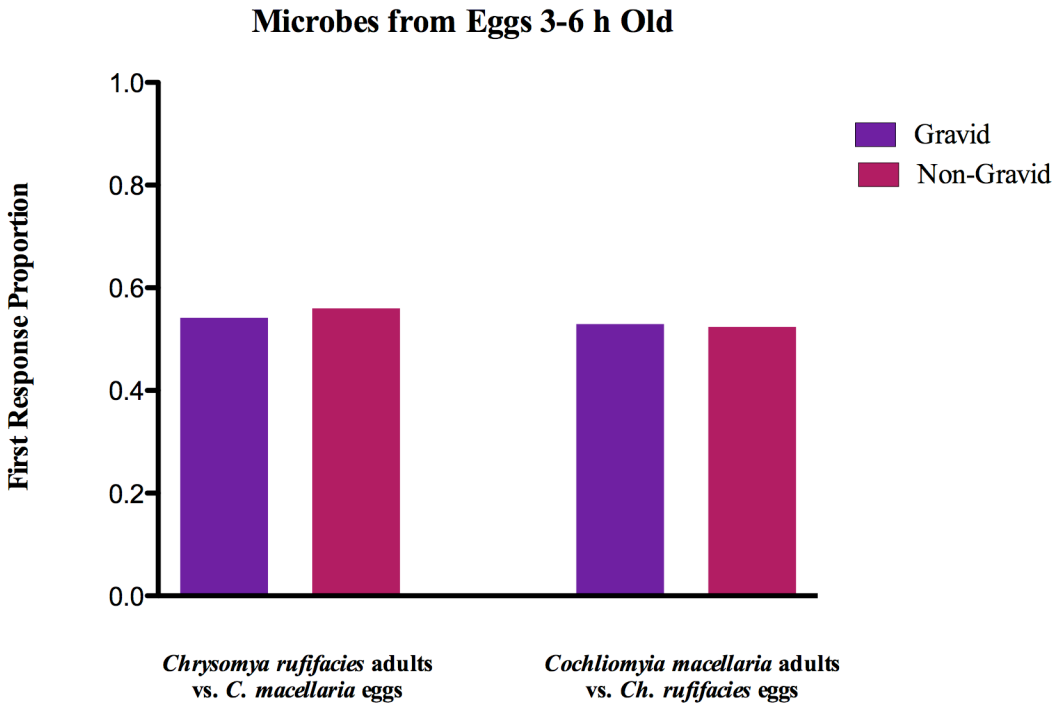

Fig A14. Proportion of adults choosing heterospecific egg assoicated microbes as initial choice in the dual choice olfactomer * indicates significant differences.

APPENDIX B

COMPLETE PYROSEQUENCING DATA FROM *CHRYSOMYA RUFIFAICES* AND *COCHOLIOMYA MACELLARIA* EGG ASSOICATED MICROBES

Description of data

These data are all the bacteria identified from *C. macellaria* and *Ch. rufifacies* eggs aged < 3 h, 3-6 h, and 6-9 h using 454 pyrosequencing as described in Chapter IV. Table B1 lists the relative abundance of all identified genera associated with all ages of eggs, while tables B2 and B3 describe the species classified as "other" (i.e. genera representing < 1% of relative abundance) as described in Chapter IV.

Table B1. Relative abundance of bacterial genera identified using 454 pyrosequencing from all egg ages of both *C. macellaria* and *Ch. rufifacies*.

***C. macellaria* egg-associated bacterial genera**	**Relative abundance (%)**	***Ch. rufifacies* egg-associated bacterial genera**	**Relative abundance (%)**
Lactobacillus	31.66%	*Lactococcus*	34.56%
Unclassified	22.17%	*Lactobacillus*	27.83%
Vagococcus	8.51%	*Vagococcus*	11.63%
Carnobacterium	7.79%	Unclassified	10.84%
Escherichia/Shigella	5.08%	*Myroides*	4.29%
Providencia	4.46%	*Providencia*	2.49%
Morganella	3.87%	*Staphylococcus*	2.40%
Staphylococcus	2.66%	*Ignatzschineria*	1.03%
Leuconostoc	2.52%	*Sphingobacterium*	0.88%
Kurthia	2.43%	*Delftia*	0.83%
Pseudochrobactrum	1.59%	*Morgenella*	0.71%
Enterococcus	1.12%	*Enterococcus*	0.61%
Lactococcus	0.80%	*Carnobacterium*	0.15%
Buttiauxella	0.56%	*Leuconostoc*	0.15%
Proteus	0.56%	*Proteus*	0.15%
Yaniella	0.50%	*Bacteroides*	0.14%
Hafnia	0.33%	*Comamonas*	0.14%
Brevundimonas	0.26%	*Pseudochrobactrum*	0.14%
Bacteroides	0.25%	*Raoultella*	0.14%
Acinetobacter	0.24%	*Chryseobacterium*	0.11%
Corynebacterium	0.22%	*Serratia*	0.11%
Clostridium	0.21%	*Microbacterium*	0.09%
Brochothrix	0.20%	*Azospirillum*	0.08%
Ignatzschineria	0.17%	*Propionibacterium*	0.08%
Salinicoccus	0.17%	*Leucobacter*	0.05%
Streptococcus	0.17%	*Acinetobacter*	0.03%
Wohlfahrtiimonas	0.17%	*Dysgonomonas*	0.03%
Jeotgalicoccus	0.16%	*Hafnia*	0.03%
Myroides	0.13%	*Kocuria*	0.03%
Devosia	0.10%	*Polaromonas*	0.03%
Alistipes	0.07%	*Pseudorhodoferax*	0.03%
Aeromonas	0.05%	*Bradyrhizobium*	0.02%
Macrococcus	0.05%	*Clostridium*	0.02%
Sphingobacterium	0.05%	*Dorea*	0.02%
Sphingomonas	0.05%	*Escherichia/Shigella*	0.02%
Alloiococcus	0.04%	*Faecalibacterium*	0.02%
Pseudomonas	0.04%	*Methylobacterium*	0.02%
Ureibacillus	0.04%	*Nitrobacter*	0.02%

Anaerococcus	0.03%	*Pelomonas*	0.02%
Brevibacterium	0.03%	*Pseudomonas*	0.02%
Paracoccus	0.03%	*Pseudonocardia*	0.02%
Propionibacterium	0.03%	*Sphingomonas*	0.02%
Raoultella	0.03%	*Streptococcus*	0.02%
Roseomonas	0.03%	*Veillonella*	0.02%
Serratia	0.03%	*Yaniella*	0.02%
TM7_genera_incertae_sedis	0.03%		
Ulvibacter	0.03%		
Weissella	0.03%		
Abiotrophia	0.01%		
Alcaligenes	0.01%		
Anaerobacter	0.01%		
Azospirillum	0.01%		
Blastomonas	0.01%		
Brachybacterium	0.01%		
Bradyrhizobium	0.01%		
Chryseobacterium	0.01%		
Coprobacillus	0.01%		
Dysgonomonas	0.01%		
Facklamia	0.01%		
Faecalibacterium	0.01%		
Fastidiosipila	0.01%		
Kaistia	0.01%		
Methylobacterium	0.01%		
Perlucidibaca	0.01%		
Porphyromonas	0.01%		
Schlegelella	0.01%		
Sphingopyxis	0.01%		
Sporacetigenium	0.01%		
Variovorax	0.01%		

Table B2. Relative abundance of bacterial genera on *Cochliomyia macellaria* eggs as identified by 454 pyrosequencing.

Genera on < 3 h eggs	Relative Abundance (%)	Genera on 3-6 h eggs	Relative Abundance (%)	Genera on 6-9 h eggs	Relative Abundace (%)
Lactobacillus	32.25%	*Escherichia/Shigella*	17.38%	*Lactobacillus*	54.81%
Unclassified	11.83%	*Lactobacillus*	10.91%	*Carnobacterium*	12.89%
Vagococcus	11.83%	*Kurthia*	8.31%	*Vagococcus*	6.52%
Morganella	11.13%	*Staphylococcus*	7.63%	*Leuconostoc*	3.78%
Carnobacterium	5.81%	*Providencia*	7.18%	*Providencia*	1.83%
Other	0.04%	*Yaniella*	1.66%	*Other*	20.18%
Providencia	4.09%	*Lactococcus*	1.30%	*Unclassified*	13.62%
Pseudochrobactrum	2.42%	*Other*	7.72%	*Enterococcus*	0.88%
Leuconostoc	1.06%	*Unclassified*	37.90%	*Morganella*	0.88%
Enterococcus	0.00%	*Vagococcus*	0.90%	*Staphylococcus*	0.80%
Buttiauxella	0.00%	*Proteus*	0.76%	*Buttiauxella*	0.72%
Proteus	0.92%	*Clostridium*	0.72%	*Lactococcus*	0.53%
Brevundimonas	0.88%	*Salinicoccus*	0.58%	*Brochothrix*	0.42%
Hafnia	0.84%	*Jeotgalicoccus*	0.54%	*Bacteroides*	0.38%
Lactococcus	0.79%	*Ignatzschineria*	0.49%	*Corynebacterium*	0.31%
Staphylococcus	0.53%	*Acinetobacter*	0.45%	*Hafnia*	0.23%
Wohlfahrtiimonas	0.53%	*Myroides*	0.45%	*Alistipes*	0.19%
Corynebacterium	0.26%	*Streptococcus*	0.45%	*Proteus*	0.19%
Devosia	0.26%	*Bacteroides*	0.40%	*Pseudochrobactrum*	0.15%
Acinetobacter	0.22%	*Enterococcus*	0.31%	*Acinetobacter*	0.11%
Brochothrix	0.18%	*Aeromonas*	0.18%	*Alloiococcus*	0.11%
Pseudomonas	0.13%	*Macrococcus*	0.18%	*Streptococcus*	0.11%
Ignatzschineria	0.09%	*Carnobacterium*	0.13%	*Ureibacillus*	0.11%
Paracoccus	0.09%	*Corynebacterium*	0.13%	*Anaerococcus*	0.08%
Sphingobacterium	0.09%	*Morganella*	0.13%	*Devosia*	0.08%
Sphingomonas	0.09%	*Brevibacterium*	0.09%	*Sphingomonas*	0.08%
TM7_genera_incertae_sedis	0.09%	*Raoultella*	0.09%	*Propionibacterium*	0.04%
Abiotrophia	0.04%	*Roseomonas*	0.09%	*Schlegelella*	0.04%
Alcaligenes	0.04%	*Serratia*	0.09%	*Sphingopyxis*	0.04%
Azospirillum	0.04%	*Sphingobacterium*	0.09%	*Weissella*	0.04%
Blastomonas	0.04%	*Ulvibacter*	0.09%	*Wohlfahrtiimonas*	0.04%
Bradyrhizobium	0.04%	*Anaerobacter*	0.04%		
Chryseobacterium	0.04%	*Brachybacterium*	0.04%		
Faecalibacterium	0.04%	*Coprobacillus*	0.04%		
Fastidiosipila	0.04%	*Dysgonomonas*	0.04%		
Kaistia	0.04%	*Facklamia*	0.04%		
Methylobacterium	0.04%	*Porphyromonas*	0.04%		
Perlucidibaca	0.04%	*Propionibacterium*	0.04%		
Variovorax	0.04%	*Sporacetigenium*	0.04%		

Weissella	0.04%
Yersinia	0.04%

Table B3. Relative abundance of bacterial genera on *Chrysomya rufifacies* eggs as identified by 454 pyrosequencing.

Genera on < 3 h eggs	Relative Abundance (%)	Genera on 3-6 h eggs	Relative Abundance (%)	Genera on 6-9 h eggs	Relative Abundace (%)
Lactobacillus	45.69%	*Lactococcus*	42.07%	*Lactococcus*	76.84%
Vagococcus	19.05%	*Lactobacillus*	23.83%	*Lactobacillus*	24.40%
Other	17.32%	*Myroides*	15.99%	*Unclassified*	18.15%
Lactococcus	12.57%	*Other*	11.12%	*Vagococcus*	15.20%
Unclassified	12.48%	*Unclassified*	7.66%	*Other*	13.68%
Staphylococcus	2.22%	*Vagococcus*	4.86%	*Providencia*	7.54%
Delftia	2.00%	*Providencia*	1.09%	*Staphylococcus*	5.36%
Sphingobacterium	1.15%	*Staphylococcus*	1.03%	*Ignatzschineria*	3.01%
Providencia	0.80%	*Ignatzschineria*	0.79%	*Morganella*	1.89%
Myroides	0.80%	*Morgenella*	0.73%	*Sphingobacterium*	1.53%
Enterococcus	0.67%	*Proteus*	0.49%	*Enterococcus*	1.41%
Leuconostoc	0.44%	*Sphingobacterium*	0.36%	*Comamonas*	0.53%
Carnobacterium	0.44%	*Bacteroides*	0.30%	*Pseudochrobactrum*	0.53%
Propionibacterium	0.22%	*Delftia*	0.24%	*Chryseobacterium*	0.41%
Microbacterium	0.22%	*Serratia*	0.18%	*Raoultella*	0.41%
Ignatzschineria	0.18%	*Acinetobacter*	0.06%	*Delftia*	0.35%
Morganella	0.13%	*Bradyrhizobium*	0.06%	*Azospirillum*	0.29%
Bacteroides	0.13%	*Dysgonomonas*	0.06%	*Serratia*	0.24%
Raoultella	0.09%	*Enterococcus*	0.06%	*Leucobacter*	0.18%
Pseudorhodoferax	0.09%	*Hafnia*	0.06%	*Myroides*	0.12%
Proteus	0.09%	*Streptococcus*	0.06%	*Polaromonas*	0.12%
Kocuria	0.09%			*Acinetobacter*	0.06%
Veillonella	0.04%			*Bacteroides*	0.06%
Pseudonocardia	0.04%			*Clostridium*	0.06%
Pseudomonas	0.04%			*Microbacterium*	0.06%
Pelomonas	0.04%			*Nitrobacter*	0.06%
Methylobacterium	0.04%			*Sphingomonas*	0.06%
Hafnia	0.04%			*Yaniella*	0.06%
Faecalibacterium	0.04%				
Escherichia/Shigella	0.04%				
Dysgonomonas	0.04%				
Dorea	0.04%				

VITA

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