LARVAL COMPETITION OF *HERMETIA ILLUCENS* (L.) (DIPTERA: STRATIOMYIDAE) AND THEIR EFFECTS ON LIFE HISTORY TRAITS, INCLUDING ADULT MATING SUCCESS

A Dissertation

by

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ABSTRACT

Ephemeral resources are a nutritious source, distributed randomly (temporally and spatially), and promote species diversity. Larval aggregations on ephemeral resources are common within dipteran species and typically lead to interspecific and intraspecific competition. This study aimed to investigate intraspecific competition (i.e., larval competition) and their effects on the life history traits and mating success of the black soldier fly *Hermetia illucens*, (L.) Diptera: Stratiomyidae. Previous studies have not thoroughly investigated intraspecific competition of *H. illucens* and their life history traits on an industrial scale. Also, knowledge of egg production from different sized black soldier fly adults have been limited. In this study, the first objective was to determine if different larval population densities yielded significant results. The next objective was to validate if color influenced mate choice. The last objective was to determine if large and small adults differed in successful matings and egg production. For the first objective, the lowest larval density of 500 larvae/4L produced larger adults compared to the highest larval density of 2000 larvae/4L. In addition, low larval densities were significantly different in development time and size across all life stages compared to the highest larval density. In the second objective of the validation study, marking techniques of four various colors did not affect mate choice of marked individuals. Finally, large male adults displayed the most mating successes and level of aggression compared to small males. Large females produced more eggs, but hatch rate slightly decreases. The variability of egg production was prominent in mixed-sized adult treatments compared to large adult treatments only, indicating that such variability could result from various other factors such as size differences and mixed ages. Such information is valuable to the mass production industry of *H. illucens* in order to predict egg viability output with each generation.

DEDICATION

I dedicate this dissertation to my late mother, Shirley Johnson-Jones. She stressed the importance of education and made me promise her that I would go further than she could accomplish when she was my age. I did not believe I would get this far without her spiritual guidance. I love you, mom. I did it!

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NOMENCLATURE

BSF	Black soldier fly
BSFL	Black soldier fly larvae
d	Day(s)
hr	Hour(s)
L:D	Light: Dark cycle
RH	Relative humidity

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1.1 The importance of carrion

Organisms, ranging from insects to vertebrates, utilize carrion as a nutrient source (McKinnerney 1978, Braack 1987, Parmenter and MacMahon 2009). For example, protein-deprived blow flies, *Lucilia sericata,*(Meigen) (Diptera: Calliphoridae) outnumber protein-fed blow flies on carrion indicating their dependence on such resources for egg production (Wall and Fisher 2001). Other arthropods that do not use carrion as their primary resource, such as butterflies (Lepidoptera), have also been shown to exploit its resources. For example, Hall and Willmott (2000) attributed a higher presence of adult male butterflies (Lepidoptera: Riodinidae) on carrion than flowers and mud because the sodium content in carrion stabilized their metabolic rates for rapid flight, which in turn helped locate mates for reproductive success. Vertebrates, such as spotted hyenas (*Crocuta crocuta*) (Erxleben) increased their scavenging behavior for carrion whenever their prey population (e.g., gazelles, wildebeests) temporarily declined (Cooper et al. 1999). Schlacher et al (2013) reported over five different species of vertebrate scavengers on fish carrion, ranging from ghost crabs (*Ocypode* spp.) to silver gulls (*Chroicocephalus novaehollandiae*) (Stephens).

The introduction of vertebrate carrion into an ecosystem promotes species diversity, especially among arthropod communities (Goldberg and Novoplansky 1997, Towne 2000, Smith and Baco 2003, Beasley et al. 2012, Barton et al. 2013). In Austria, a carrion succession study conducted from May to August, and August to November, found over 20 different insect families associated with two pig carcasses (Grassberger and Frank 2004). The most abundant arthropod species associated with vertebrate carrion belong to flies (Diptera), mites (Acari), and beetles

(Coleoptera) (Braack 1981, Barton et al. 2014). Another carrion succession experiment from November to May in Australia reported over 20 mite species, and over 85 beetle species from 18 eastern grey kangaroo (*Macropus giganteus*) (Shaw) carcasses (Barton et al. 2014). In South Africa, Braack (1981) collected over 5000 dipteran individuals comprising of more than five different species located on impala (*Aepyceros* spp.) carcasses.

1.2 Carrion as an ephemeral resource

Vertebrate carrion is an ephemeral resource due to several unique characteristics. A common characteristic is that it typically decomposes at a rapid rate; as described by Payne (1965) who found that during the summer in Clemson, South Carolina, 90.0% of pig carrion was reduced by arthropods to skeletal remains within six days. In the Czech Republic, rat carrion exposed to insects in forested and meadow habitats decomposed within a month during three different seasons (Kočárek 2003). In spring (May-June) and autumn (October-November), over 50.0% of the total rat biomass was removed within an average of 35 days. In the summer (July-August), over 60.0% of the total rat biomass was removed within an average of 26 days. However, there are circumstances where large carrion can take more than a year to decompose. For example, Smith and Baco (2003) stated that it will take more than 1.5 years for the soft tissue of whales to be digested by scavengers.

As an ephemeral resource, vertebrate carrion is also high in nutrients. Parmenter and MacMahon (2009) examined 11 types of vertebrate species ranging from mule deer (*Odocoileus hemionus*) (Rafinesque) and least chipmunk (*Tamias minimus*) (Bachman), to the Sage sparrow (*Amphispiza belli*) (Cassin) and Northern leopard frog (*Lithobates pipiens*) (Schreber), and found that their nutrient composition includes inorganic compounds such as nitrogen (est. 7.0-12.0%),

calcium (est. 3.0-7.0%), phosphorus (est. 1.6-4.0%), and many other inorganic compounds. Over a 10 month decomposition period (July-May) in Wyoming, 15 rainbow trout (*Oncorhynchus mykiss*) (Walbaum) and 15 pintail duck (*Anas acutas*) (L.) carrion lost more than 60.0% of their initial potassium, sodium, and nitrogen concentrations that were recycled back into the aquatic ecosystem (Parmenter and Lamarra 1991). The authors suggest that the significant element losses serve as a nutrient sink, or pools of nutrients within a habitat that have accumulated over time, for the aquatic ecosystem.

Furthermore, vertebrate carrion is typically distributed randomly (temporally and spatially) in the environment, as its availability depends on the cause and location of its mortality such as predation, or disease (DeVault et al. 2003). For example, less than 5.0% of reindeer (*Rangifer tarandus*) (L.) die from predation in northern Scandinavia (Oksanen and Oksanen 2000, DeVault et al. 2003). But reindeer free from predators in South Georgia Island often die from starvation and falling accidents ((Tyler and Oritsland 1999) as translated by (DeVault et al. 2003)). Overall, the causes of mortality depend on the presence of natural enemies in native habitats. However, carrion occurrence can be predictable (i.e., resource pulses). For example, Alaskan salmon (*Oncorhynchus* spp.) migration in autumn serves as a resource pulse for coastal minks (*Mustela vison*) (Schreber) because once the adult salmon spawn, they die (Ben-David et al. 1997). Fifty percent of salmon remains can be transferred to riparian zones by black bears (*Ursus americanus*) and wolves (Hocking and Reimchen 2006), which allows more than sixty species of invertebrate scavengers, such as Diptera and Coleoptera, to inhabit the carcasses (Hocking et al. 2009).

1.3 Resource pulse

Resource pulses are events of resource superabundance that combine low frequency, short duration, and high magnitude, and can be beneficial to other organisms (Holt 2008, Yang et al. 2008). For example, periodical cicadas (*Magicicada* spp.) (Davis) (Hemiptera: Cicadidae) emerge from the ground every 17 years to locate mates (Yang 2004). Once they die, their carcasses decompose in the soil and increase soil ammonium (by 412.0%) and nitrate (by 199.0%) content, which stimulates plant productivity (Yang 2004). In particular, the American bellflower (*Campanulastrum americanum*) produced seeds that were 9.0% larger than plants not exposed to cicada carcasses; and their leaves contained 12.0% more nitrogen (Yang 2004). Wilmers et al (Wilmers et al. 2003) studied over a three year period that from early January to mid-February, hunters in Yellowstone National Park provide increased elk (*Cervus elaphus*) (L.) carrion biomass (33,203.0 ± 993.0 kg) to scavengers, compared to elk carrion biomass provided by grey wolves (*Canis lupus*) (L.) (13,220.0 ± 383.9 kg). When there is an abundance of resources present within a certain habitat, consumers will inhabit that area and compete with one another in order to utilize the nutrients present within the carrion (Trumbo 1992).

1.4 Interspecific competition

Interspecific competition that involves two or more species competing for a limiting resource frequently occurs for carrion (Firbank and Watkinson 1985). Such competition increases as the arthropod community diversifies throughout carrion decomposition (Braack 1987). For example, *Chrysomya albiceps* (Wiedemann) (Diptera: Calliphoridae) are predators of other Dipteran species (Faria and Godoy 2001). They, along with *Chrysomya putoria* (Wiedemann), *Cochliomyia macellaria* (Fabricius) and *Chrysomya megacephala* (Fabricius) (Diptera: Calliphoridae) utilize

carrion for feeding and breeding purposes, and often engage in interspecific competition (Faria et al. 1999). A laboratory study in Brazil (Faria et al. 1999) found that third instars of *C. albiceps* preyed on the other third instar blow fly species, with *C. macellaria* having the highest predation rate (77.5% in 120 minutes). However, in a similar study (Faria and Godoy 2001), where *C. macellaria* wasn't present, *C. putoria* had the highest predation rate (72.5% in 120 minutes) compared to *C. megacephala* (15.0% in 120 minutes). The predation rate of *C. albiceps* suggests that they may consume other organisms during periods of starvation (Faria et al. 1999).

1.5 Intraspecific competition

Although most carrion ecology research focuses on interspecific competition, intraspecific competition, which involves only one species, is also prevalent within the carrion fly community. Interspecific competition focuses on limiting the number of competitively inferior species to maximize resource availability for the dominant species, while intraspecific competition (involving only one species) focuses on reducing the population size of a single species of interest to promote resource availability for their immature stages (Fuller 1934). For example, a study compared intraspecific competition of various larval *L. sericata* populations originating from Spain, the United Kingdom, and a hybrid population from both countries, and found that all three populations had lower survival rates (40-70% mortality) with increasing population density (Martínez-Sánchez et al. 2007). Mean adult sizes declined from 1.3 mm to 0.9 mm as the population density increased, with females being larger than males until both sexes reached about the same size as population density increased (Martínez-Sánchez et al. 2007). The adult size differences suggest that high levels of competition occurs with higher population densities, which limits resource availability and reduces the size of consumers.

1.6 Population oscillation

A population will have predictable oscillation patterns when maintained in a constant environment (Nicholson 1950). The fluctuations within a population are often caused by multiple species competing for natural resources. When resources are abundant, organisms will colonize the area to feed and promote their development. However, when the population goes beyond the capacity that the resources can provide, competition increases and the particular species of a given population will decline if they do not have the ability to compete. For example, Goodbrod and Goff (1990) investigated the cannibalistic behavior of larval *Chrysomya rufifacies* (Macquart) on larval *C. megacephala*, and larval and puparial mortality of both species when reared in beef liver cultures at seven different population densities. *Chrysomya megacephala* larval and puparial mortality decreased until the population density reached a threshold of 8 larvae/liver, but the mortality rate increased again once it reached the highest population density of 40 larvae/liver, suggesting a population oscillation within different larval densities. *Chrysomya rufifacies* exhibited similar results, only that the larval and puparial mortality rates declined until it reached a population threshold at 10 larvae/liver. When both species were placed in the same containers without liver resources, second to third instar larvae of *C. rufifacies* consumed *C. megacephala* as an alternative food source in order to complete their development and avoid high larval and puparial mortality rates (Goodbrod and Goff 1990). Although both blow flies were reared with limited or no resources, the strategy of predation from *C. rufifacies* provides temporary stress relief by locating an alternative food resource.

1.7 Non-consumptive effect

The presence of predators near resources can affect prey in two ways: direct consumption of prey, or alter prey behavior, which is also known as a non-consumptive effect (NCE) (Weissburg et al. 2014). Currently, there are no studies linking NCE to the carrion community. However, Fischer et al (2012) of Argentina reared larval *Culex pipiens* (L.) (Diptera: Culicidae) in the presence of predaceous adult *Notonecta sellata* (Latreille) (Hemiptera: Notonectidae), and found that *N. sellata* consumed the second and third instars of *C. pipiens* within 24 hours. The mean development time of *C. pipiens* reared in the presence of *N. sellata* ranged from 1.8-4.3 days compared to the control *C. pipiens* population that averaged 1.5-3.5 days, suggesting that *C. pipiens* spent more energy avoiding the predator, resulting in less energy cost for obtaining nutrients for development (Fischer et al. 2012).

1.8 Larval aggregation

Traditional hypotheses examining competition suggest that long-term competition will result in the eradication of one or more species until a "winner" is left standing (Hardin 1960, DeBach 1966, Hanski 1987). However, the strategy of larval aggregation has been used within the carrion fly community where they inhabit various parts of carrion and coexist among one another to exploit resources (Ives 1991, Kouki and Hanski 1995, Boulay et al. 2013). For example, in double species cultures of *C. putoria* and *C. macellaria* (Fabricius) (Diptera: Calliphoridae), larval survival rates increased from 5.0% to 45.0% as the population density increased from 200 (100 of each spp.) to 1,600 (800 of each spp.) (Reis et al. 1999). This suggests that the aggregation of both species increases efficiency of the feeding process from the digestive enzymes and numerous mouth hooks (Goodbrod and Goff 1990). However, when examining the survival rates of each species in double

cultures, *C. macellaria* is a competitively inferior species in the presence of *C. putoria* (Reis et al. 1999). As long as the inferior competitor is not clumped in the same sites as the superior competitor, this ensures that it can survive in breeding sites where the other species is absent or at low density (Atkinson 1985).

1.9 Past research linking resource availability to reproductive success

Previous studies have investigated the correlation among availability of resources to reproductive success. The larval or adult nutrition derived from resources is a primary factor that defines the organism's preferred environment and physiological activity (Raubenheimer 2010). In regards to reproduction, many studies have investigated how male nutrition affects mating success because more energy is used to attract a female. Chapman et al (1996) noted that male *Drosophila melanogaster* (Meigen) (Diptera: Drosophilidae), had a higher mating frequency when reared on a balanced diet. Fricke et al (2008) reported that *Drosophila* males fed a resource with 20.0% yeast content are less likely to successfully re-mate with non-virgin females. Engels and Sauer (2007) studied larval nutrition of male scorpionflies, *Panorpa vulgaris* (Imhoff & Labram) (Mecoptera: Panorpidae), and reported that starved larvae become less effective reproductive competitors for females because their low weight decreased the size of their salivary glands. Male scorpionflies' produce salivary secretions during copulation and feed the females to prolong copulation duration (as translated by (Engels and Sauer 2007) from original source (Kaltenbach 1978)). One study found that the dietary preferences for field crickets *Teleogryllus commodus* (Walker) (Orthoptera: Gryllidae) were varied based on the sex of the insect (Maklakov et al. 2008). The females significantly consumed more diets with higher protein concentrations ($p < 0.03$), while males preferred diets higher in carbohydrates for energy to make mating calls ($p < 0.001$) (Maklakov et

al. 2008). This suggests that when conducting nutritional studies for a species of interest, it is important to consider both sexes to understand essential nutrients that are needed between them.

There have been studies suggesting that there is a trade-off between mating success and longevity from lack of resources (Weindruch and Walford 1988, Masoro 2005, Lee et al. 2008), where organisms will either focus their energy on producing offspring, but die immediately after mating and oviposition; or they will focus their energy on their development, extending their lifespan yet limiting reproduction. However, other studies (Maklakov et al. 2008, Grandison et al. 2009, Maklakov et al. 2009) have found conflicting results, suggesting that reproduction and longevity can coexist under the right nutritional conditions. It was concluded that sexual maturation and lifespan may proceed at different rates in males and females of a given species (Simpson and Raubenheimer 2012).

1.10 The black soldier fly

The black soldier fly, *Hermetia illucens,* (L.) (Diptera: Stratiomyidae) represents a unique model for exploring questions related to intraspecific competition for ephemeral resources and resulting impact on mating success. Their rise in popularity has been due to an interest in their applications in waste management (Sheppard et al. 1994), alternative food source for other organisms (Newton et al. 1977, Bondari and Sheppard 1981, St-Hilaire et al. 2007) , and forensic entomology (Tomberlin et al. 2004, Pujol-luz et al. 2008, Vanin et al. 2011). Their dense presence in animal waste during their larval stages has been documented to significantly reduce the larval populations of the pest species *Musca domestica* (L.) (Diptera: Muscidae) (Bradley and Sheppard 1984, Sheppard et al. 1994); and they aid in the decline of nitrogen and phosphorus content in poultry and dairy manure (Myers et al. 2008). This knowledge can help solve the issues of large manure

accumulations surrounding concentrated livestock farming operations. These insects can also be used as feed for a variety of livestock such as poultry, fish, and swine (Newton et al. 1977, Bondari and Sheppard 1981, St-Hilaire et al. 2007). If the pre-pupae are dried, their protein content can be compared to soybean or meat and bone meal (Newton et al. 1977, Sheppard et al. 1994).

As detritivores (organisms that consume dead, organic matter), larvae of black soldier flies have been associated with pig carrion, and human remains (Tomberlin et al. 2004, Martínez-Sánchez et al. 2011). In Europe, black soldier fly pre-pupae and pupae were reported to be found on human remains at the advanced stage of decay (Martínez-Sánchez et al. 2011, Vanin et al. 2011), indicating that they are late colonizers. However, Tomberlin et al (2004) reported black soldier fly larvae present on pig carrion as early as a week after distribution into the environment, suggesting that they are not always late colonizers.

The black soldier fly is a wasp-like fly that has three generations per year in the southeastern United States (Tomberlin et al. 2002). Females lay a single clutch (320.0-680.0 eggs) that hatch in 102.0-105.0 hours at 24.0°C (Booth and Sheppard 1984). Larvae feed on many types of decomposing organic matter such as rotting fruits and animal tissue (Tingle et al. 1975); however, the black soldier fly can be maintained on the grain-based, Gainesville House Fly Diet (50.0% wheat bran, 20.0% corn meal, and 30.0% alfalfa meal (Hogsette 1992)) (Sheppard et al. 2002). Sheppard et al (1994) stated that the larvae need to store a large amount of fat in order to survive as adults. The black soldier fly larval stage can last from 22-24 d at 27.0°C (Tomberlin et al. 2002). Once they reach the pre-pupal stage, they stop consuming food and disperse to a safe location for pupation, which takes approximately two weeks (10-14 days) at 27-30°C (Sheppard et al. 2002). As adults, these flies no longer require nutrients, only water, as those nutrients

acquired during larval development are used to mate and ensure the success of their offspring (Tomberlin et al. 2002).

Black soldier fly adults are known to perform lekking behavior (Tomberlin and Sheppard 2001). Lekking behavior has been described in a closely related species, *Hermetia comstocki* (Williston) (Diptera: Stratiomyidae) (Alcock 1990). The males rest on the surfaces of *Agave palmeri* (Engelm), until the arrival of another male provokes a "battle," where the resting male competes with the invading male and they spiral about 0.5 to 1.5 meters above the resting male's leaf territory (Alcock 1990). Once the height of the battle has been reached, the "winning" male fly will return to the resting leaf and the "loser" flies away. The resting male then ascends and grabs onto a passing female mid-air. Copulation takes place with the male facing the opposite direction of the female while they remain joined by their genitalia as they descend on the leaf (Tomberlin and Sheppard 2001). The impact of male size on combat success during lekking still remains unknown. However, it has been suggested that the larger the male, the higher the chance of "winning" the male-to-male competition that results in female selection (Tingle et al. 1975, Alcock 1990). However, this aspect of their mating behavior remains poorly understood, especially among *H. illucens.*

1.11 Overview and objectives

Many studies have examined the effects of blow fly larval population densities on the rate of competition, but few have conducted similar experiments with black soldier flies. In addition to the lack of information, very few studies have explored the mating behavior of black soldier flies (Tingle et al. 1975, Alcock 1990, Tomberlin and Sheppard 2001), or the influence of larval population densities on competition and securing a mate. If black soldier flies show a significant

change in size, development time, and mating behavior based on their available food resources obtained during their larval stages, then this research can launch future studies to understand how environmental factors affect their developmental rates that are often linked to waste management and even forensic entomology. This proposal will tie intraspecific competition to mating behavior of black soldier flies by providing detailed information on the following: black soldier flies' mating behavior and egg production. The specific objectives of this study are defined below:

i. Determine the larval life-history traits of black soldier flies at different larval densities.

Rationale: Competition for food resources is a critical component of population structure within a habitat because it determines the exploitation of resources for a given population (Ullyett 1950). The first objective will examine the impact of larval population densities on survivorship, longevity, and size of the black soldier fly. There will be four population densities to examine, and each one is based on the number of individual black soldier fly larvae within the categories. Hypotheses tested include:

H_0: Black soldier fly larval development time, survivorship, and size are independent of their larval population density.

H_A: Black soldier fly larval development time, survivorship, and size are dependent of their larval population density.

ii. Determine if color impacts mate choice and egg production by black soldier flies.

Rationale: Many studies for monitoring insect mating behavior, as well as mark-recapture of insect pests, use fluorescent dusts. In addition, marking materials that are non-toxic, adhesive, and lightweight are important for conducting behavioral research. Currently, there are limited studies that evaluate the effectiveness of marking techniques on the black

soldier fly. Such data are imperative for future research involving *H. illucens*. In addition, it is unclear whether marking black soldier flies affect egg production. The use of four different acrylic paint colors will be explored in order to determine if color affects mate choice and/or egg production. Hypotheses tested include:

H_0: Color does not influence BSF mate choice and egg production.

H_A: Color influences BSF mate choice and egg production.

iii. Determine how mating success is impacted by black soldier fly larvae reared in different population densities.

Rationale: Although not fully understood, it has also been suggested that for *Hermetia comstocki,* a similar species to *H. illucens* in terms of behavior, that size is determined by the amount of food consumed in their larval stages (Alcock 1990). However, it is possible that intraspecific competition by larval population densities can affect the ability to obtain a mate. Once the immature flies reach their adult stages, the resources they have obtained from their larval development will be converted to energy that will be used during their mating behavior. Male black soldier flies will most likely spend more of their energy competing with other males and obtaining a female for copulation. Male black soldier flies may show a significant change in their mating behavior based on the level of competition during their larval stages, and may not be able to cross-compete with males and cross-mate with females reared within different population densities. Hypotheses tested include:

H_0: Black soldier fly males will not successfully mate with females reared from different population densities.

H_A: Black soldier fly males will successfully mate with females reared from different population densities.

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CHAPTER II

IMPACT OF LARVAL COMPETITION ON LIFE-HISTORY TRAITS OF THE BLACK SOLDIER FLY, *HERMETIA ILLUCENS* (L.) (DIPTERA: STRATIOMYIDAE)[1]

The black soldier fly, *Hermetia illucens* (L.) (Diptera: Stratiomyidae), is economically important due to its use in waste management and as an alternative protein source for livestock, poultry, and aquaculture. While industry promotes mass production of the black soldier fly, little is known about the impact of larval competition on development time, resulting immature and adult weight, or adult longevity. The goal of this research was to examine the life-history traits of black soldier flies when reared at four densities (500, 1000, 1500, and 2000 larvae/4 L container) provided 54 g Gainesville diet at 70% moisture (feed rates of 0.027, 0.036, 0.054, and 0.108 g) every other day. Results were as expected with the lowest larval density (500) producing heavier individuals (by 26%) than the greatest larval density (2000) across all life stages. In addition to weights, larvae reared at the lowest density developed 63% faster than those reared at the greatest density. In regards to pupal development time, those reared at the lowest larval density developed 3% slower than the greatest density. A 21% difference between the two extreme densities was found in survivorship to prepupal stage, with the lowest larval density having the greatest survivorship (92%) compared to the greatest larval density (70%). All densities displayed over 90% adult emergence rates. Such information is vital for optimization of the process of converting waste products to protein at an industrial scale with the black soldier fly.

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2.1 Introduction

The black soldier fly (BSF), *Hermetia illucens* (L.) (Diptera: Stratiomyidae), is wasp-like in appearance with three generations per year in the southeastern United States (Sheppard et al. 1994). Adults lay a single egg clutch (320-689 eggs) and individual eggs hatch in 102-105 h at 24°C (Sheppard et al. 2002, Tomberlin et al. 2002). Larvae feed on many types of decomposing organic matter such as rotting fruits and animal tissue (James 1935, Tingle et al. 1975), food waste (Nguyen et al. 2015), poultry manure (Sheppard 1983), swine manure (Newton et al. 2005), dairy manure (Li et al. 2011), and human feces (Banks et al. 2014); however, the BSF can be maintained on a grain-based Gainesville house fly, *Musca domestica* L. (Diptera: Muscidae), diet (50.0% wheat bran, 20.0% corn meal, and 30.0% alfalfa meal) (Hogsette 1992) in colony (Sheppard et al. 2002). Black soldier fly larval development can last from 22-24 d at 27.0°C (Tomberlin et al. 2002), and pupation can be completed in approximately two weeks (10-14 d) (Sheppard et al. 2002). It is hypothesized that adult BSF rely on nutrients acquired during larval development (Tomberlin et al. 2002) as adult flies do not need to feed.

Research interest in the BSF increased over the past 20 years due to its applications as a green technology in managing wastes associated with confined animal facilities (Tingle et al. 1975, Sheppard et al. 1994) and food consumption (i.e., kitchen waste) (Nguyen et al. 2015). Manure decomposition from confined animal facilities has proven to be a major contributor to compounds, such as ammonia, which are potential threats to human health (FAO 2009). In addition to manure waste, on a global scale, humans produce about 1.3 billion tons of food waste per year (FAO 2011), resulting in an increase in noxious landfill emissions (i.e., methane, carbon dioxide) that can also endanger human health (FAO 2013). In a global 50-year study, food waste mass grew from 540 Mt to 1.6 Gt, in association to greenhouse gas emissions dramatically increased from 680 Mt to

2.2 Gt CO_2e (Porter et al. 2016). One possible solution is to utilize BSF to aid in the recycling of food waste to help reduce food waste and manure emissions.

With confined animal facilities, such as poultry operations, dense populations of BSF larvae have been documented to significantly reduce (94-100%) associated house fly populations (Bradley and Sheppard 1984, Sheppard et al. 1994); additional benefits of BSF larvae include the reduction of nitrogen (30-50%) and phosphorus (61-70%) content as shown in dairy manure (Myers et al. 2008) and human feces (Banks et al. 2014). However, despite interest in maximizing BSF development on an industrial scale, the appropriate larval density for optimized waste management remains unknown.

Most research on the BSF has been conducted on a bench-top scale, and translation of these results to industrial scale is challenging. Myers et al. (2008) fed 300 BSF larvae four rates of dairy manure (27, 40, 54, and 70 g/day) and determined prepupal and adult weights were heaviest for the greater feed rates (54 and 70 g). However, percent reduction in manure was greatest (~70%) at the lower feed rates. Diener et al. (2009) exposed 200 BSF larvae to different feeding regimes from 0.01-0.20 g chicken feed/larva/day (60% moisture) and reported that larvae fed 0.20 g had the greatest prepupal weight (0.063 g) and the shortest (15.9 d) development time from egg to prepupae. Larvae provided less than 0.10 g/larva/d needed more time (4-25 d) to complete development and weighed less (0.018-0.033 g) than those provided the greatest feed rate. The authors determined the feed rate of 0.1 g/larva/day could yield the optimal waste reduction and prepupal weight. Banks et al. (2014) tested two different BSF larvae feeding regimes (incremental and lump sum) of human feces at ratio of 0.1 g feces/larva/day for 10 and 100 BSF larvae per treatment, and 1 g feces/larva/day for a single larva per treatment. One feeding regiment provided waste to BSF larva(e) every 2 d for 12 d (incremental), while larvae fed the second regime were

provided 12-120 g of feces at the beginning of the experiment and allowed them to consume the amount over 12 d (lump sum). Banks et al. (2014) reported incremental feeding of BSF larvae resulted in the shortest development time (8-10 d) compared to the lump sum feeding method (10-12 d). However, larvae fed the lump sum regime were significantly larger (0.10-0.20 g) across all densities tested than those fed incrementally. Furthermore, larvae reared at the greatest density had the greatest percentage of waste reduction (54.2-54.6%) regardless of feeding regime.

In all cases, translating laboratory data to a larger, more industrial scale is difficult. The requirements for materials and production costs (Schnell et al. 2018), as well as calculating average processing rates (Piccinno et al. 2016) for mass production, are some of the factors needed to scale laboratory data to the industrial scale. The objective of the current study was to explore different densities of BSF at levels higher than previous studied and determine the resulting impact of different feed rates on associated life-history traits. Our null hypothesis was larval density (i.e., feed rate) would not impact growth parameters of the BSF.

2.2 Methods

2.2.1 Acquisition of flies

Eggs collected from a BSF colony maintained at the Forensic Laboratory for Investigative Entomological Sciences (F.L.I.E.S.) Facility at Texas A&M University (TAMU) in College Station, Texas, were used in these experiments. The colony was established in 2014 from eggs of a laboratory colony at the Coastal Plain Experiment Station, University of Georgia, Tifton, GA, USA, which originated from material collected at a poultry facility in Bacon Co., GA, USA, in 1998. At 0900 h, corrugated cardboard (8.8 x 5 x 1.2 cm) was placed on top of a screen lid attached to a plastic box (33.0 (L) x 21.6 (W) x 30.5 (D) cm) containing 100 g of the Gainesville diet

(Hogsette 1992) at 70% moisture and BSF larvae feeding on the diet to attract gravid females (Tomberlin et al. 2002). After 24 h, resulting eggs were removed from the cardboard using a 21 cm VWR® Disposable Spatula and placed in a 100 ml cup (Frontier Agricultural Services, Newark, DE, USA) with a lid and stored in a walk-in growth chamber set at 30.2 ± 0.5°C with 60 ± 5.1% RH and 14L:10D photoregime. Eggs were monitored every 12 h for eclosion and larvae were placed in a plastic 460 ml cup (Frontier Agricultural Services, Newark, DE, USA) and fed 40 g of the Gainesville diet (at 70% moisture) for 4 d (Myers et al. 2008).

2.2.2 Experiment design

Two trials were conducted for this experiment. Treatments consisted of 500, 1000, 1500, and 2000 larvae/4 L container fed 54 g Gainesville diet at 70% moisture (20 g dry diet mixed with 34 ml water) at the rate described below. Tomberlin et al. (2002) stated 300 larvae fed at 27 g (10 g mixed with 17 ml of water) of the Gainesville diet daily produced the heaviest larvae. The larval densities as previously described increased the Tomberlin et al. (2002) density by two, three, four, and five times. The lowest population density (500) was the baseline for the feed rate of 54 g as it was almost double the ratio of larvae to diet employed by Tomberlin et al. (2002). The densities translated into feed rates of diet per larva were 0.027, 0.036, 0.054, and 0.108 g.

Black soldier fly larvae (4-d-old) were transferred to 532 ml cups (Solo Company, Illinois, USA) containing 54 g of the Gainesville diet at the densities previously described. There were four replicates of each of the four densities, totaling sixteen replicates per trial. The cups containing feed and larvae were covered with white tulle fabric (Wal-Mart Stores Inc., Bentonville, AR, USA) secured by a rubber band to prevent larvae escaping and other fly species from feeding on the Gainesville diet, and placed individually in the center of a plastic pan (33.0 (L) x 21.6 (W) x 30.5

(D) cm). The plastic pans were then placed in a randomized complete block design on shelves in the walk-in growth chamber previously described. Preliminary studies indicated that mold formation occurred if 4-d-old larvae were fed every day. Therefore, larvae in all replicates of each treatment were fed 54 g Gainesville diet at 70% moisture every other day for the first 8 d to reduce the likelihood of mold. Subsequent feedings were daily. On the ninth day, the 532 ml cups were emptied into the (33.0 (L) x 21.6 (W) x 30.5 (D) cm) plastic containers also covered with tulle fabric secured by elastic cord. Feeding continued daily until 40% of the larvae reached the prepupal stage (Sheppard et al. 2002; Tomberlin et al. 2002).

2.2.3 Larval life-history traits

The largest larvae observed from each replicate were selected every 3 d, weighed individually on an Ohaus Scout® Pro Balance (Ohaus Corporation, NJ, USA), and then returned to their designated replicate. Replicates were examined daily for prepupae, which were characterized by their cuticle changing from white to brown-black (May 1961). Ten percent of total prepupae collected each day were weighed individually and placed individually in 59 ml cups covered with a lid that had a cotton ball placed through a hole (Daily Chef Food Service, Sam's Club, Bentonville, AR) and stored in the walk-in growth chamber. The collected prepupae were monitored daily for adult emergence. Time (d) required for 40% of larvae to reach the prepupal stage along with percent survivorship to the prepupal stage were recorded.

2.2.4 Adult life-history traits

Time (d) to adult emergence was recorded for each of the isolated prepupae, and emerged adults were sexed and weighed. Adults were provided water *ad libitum* via the cotton ball placed through

the cup lid (Tomberlin et al. 2002). Adult longevity was recorded daily from the date of emergence to date of mortality.

2.2.5 Statistical analysis

All statistical analyses were performed using JMP® Pro 11 software (SAS Institute Inc., Cary, NC, 1989). An analysis of co-variance (ANCOVA) was used to analyze larval weight over time. An analysis of variance (ANOVA) was used to compare larval weight, prepupal development time, prepupal weight, adult longevity, and adult weight across the four densities. ANOVA outliers were removed from each replicate using Cook's Distance calculations. Normality assumptions were checked before ANOVA implementation. All data were normal; however, some treatments did not meet equal variance assumption. Analyses were performed for taking into account unequal variance. A Tukey's HSD test was used following a significant ANOVA for separation of means ($p < 0.05$). A t-test compared the larval and adult life-history traits across sex. Two replicates of the 2000 BSF larval treatment during trial two were excluded during analysis due to high (>50%) larval mortality.

2.3 Results

2.3.1 Larval weight

No significant treatment by trial interaction ($F_{3,3} = 1.05$, $P = 0.3748$) or trial effect ($F_{1,1} = 1.92$, $P = 0.1692$) were determined for larval weight. Significant differences ($F_{3,3} = 17.06$, $P < 0.0001$) in larval weight across densities were determined. On average, larvae at the lowest density were the heaviest (0.1521 g), while those at the greatest density were the lightest (0.1301 g) (Table 2.1).

Table 2.1: Final weight (g) for black soldier fly BSF larvae when reared at different densities. Larvae were reared in a walk-in growth chamber set at 30.2 ± 0.5°C with 60 ± 5.1% RH and 14:10 (L:D) conditions.

Density	Mean Weight (g) ± Standard Error of Mean (SEM)
500	0.1521 ± 0.0057A
1000	0.1333 ± 0.0067B
1500	0.1336 ± 0.0048B
2000	0.1301 ± 0.0050B

[1]Different letters within a column indicate significant ($p < 0.05$) differences.

2.3.2 Larval development time

No significant treatment by trial interaction ($F_{3,3} = 2.07$, $P = 0.1156$) was found. However, a trial effect ($F_{1,1} = 27.95$, $P < 0.0001$) on larval development time was found. In trial two, replicates 2 and 3 of the greatest density were removed from analyses due to high larval mortality and reanalyzed. Trial data were analyzed separately to determine significant interaction. In trial one, development times were significantly different ($F_{3,31} = 149.50$, $P < 0.0001$) across all densities. Larvae reared at the lowest density developed the fastest (17.65 d) while those at the greatest density developed the slowest (33.79 d) (Table 2.2). Similar to trial one, development times were significantly different ($F_{3,25} = 58.02$, $P < 0.0001$) across all treatments. The lowest density produced shortest (21.18 d) larval development time, in contrast to the greatest density displaying the longest (41.46 d) larval development time (Table 2.2).

Table 2.2: Development time (d) of black soldier fly larvae when reared at 4 different densities. Larvae were reared in a walk-in growth chamber set at 30.2 ± 0.5°C with 60 ± 5.1% RH and 14:10 (L:D) conditions.

Trial 1		Trial 2	
Density	Days ± SEM	Density	Days ± SEM
500	17.6533 ± 0.1924A[1]	500	21.1823 ± 0.3588A
1000	21.5794 ± 0.4454B	1000	23.9983 ± 1.3589A
1500	28.4077 ± 0.7816C	1500	36.3758 ± 1.4121B
2000	33.7948 ± 0.9916D	2000	41.4655 ± 2.3917B

[1]Different letters within a column indicate significant ($p < 0.05$) differences.

2.3.3 Survivorship to pupal stage

No significant treatment by trial interaction ($F_{2,2} = 0.11$, $P = 0.9481$) or trial effect ($F_{1,1} = 0.98$, $P = 0.3311$) was found. Significant differences ($F_{2,2} = 6.79$, $p = 0.0018$) in pupal survivorship were found across densities; those reared at the lowest larval density (500) had the greatest survivorship (92.0%), while those reared at the greatest larval density (2000) had the lowest survivorship (70.93%) (Table 2.3).

Table 2.3: Larval survivorship (%) to pupal stage. Larvae were reared in a walk-in growth chamber set at 30.2 ± 0.5°C with 60 ± 5.1% RH and 14:10 (L:D) conditions. Survivorship was calculated by counting the total number of larvae that entered prepupal stage from each replicate.

Density	Survivorship (%) ± SEM
500	92.02 ± 2.9472A[1]
1000	77.62 ± 2.9472B
1500	79.73 ± 2.9472B
2000	70.93 ± 3.6096B

[1]Different letters within a column indicate significant ($p < 0.05$) differences.

2.3.4 Prepupal weight

No significant treatment by trial interaction ($F_{3,3} = 1.42$, $P = 0.2458$) or trial effect ($F_{1,1} = 2.82$, $P = 0.0988$) was found. Significant differences ($F_{7,59} = 13.25$, $P < 0.0001$) in prepupal weight were

found across densities; those reared at the lowest density (500) were the heaviest (0.1054 g), while those reared at an intermediate density of 1500 individuals were the lightest (0.0773 g). When separated by sex, weights were significantly different between males and females ($F_{1,1} = 20.88$, $P < 0.0001$); females were 15.18% heavier than males (Table 2.4).

Table 2.4: BSF prepupal weight (g) separated by sex. Prepupae were reared in a walk-in growth chamber set at 30.2 ± 0.5°C with 60 ± 5.1% RH and 14:10 (L:D) conditions.

	Sex (Mean Weight (g) ± (SEM))	
Density	M	F
500	0.1004 ± 0.0025A[1]a[2]	0.1087 ± 0.0030Aa
1000	0.0800 ± 0.0020Bb	0.0893 ± 0.0032Ba
1500	0.0702 ± 0.0025Cb	0.0830 ± 0.0038Ba
2000	0.0707 ± 0.0031BCb	0.0873 ± 0.0048Ba

[1, 2]Different capital letters within a column indicate significant ($p < 0.05$) differences across treatments, while different lowercase letters indicate significant ($p < 0.05$) differences between sexes of same treatment.

2.3.5 Pupal development

No significant treatment by trial interaction ($F_{3,3} = 2.17$, $P = 0.1021$) was found; however, a trial effect ($F_{1,1} = 20.13$, $P < 0.0001$) on pupal development time was found. In trial one, there was a significant difference ($F_{3,31} = 11.38$, $P < 0.0001$) in pupal development time across densities. Those reared at the lowest population density developed the slowest (14.45 d), yet those reared at the second greatest density (1500 individuals) developed the fastest (13.35 d). There was a significant difference ($F_{1,1} = 18.23$, $p = 0.0003$) between sexes in pupal development. Females developed slower (2 d) than males (Table 2.5).

In trial two, there were no significant differences ($F_{3,3} = 1.51$, $P = 0.2407$) in pupal development time across density. However, there was a significant difference ($F_{1,1} = 12.09$, $P =$

0.0024) between sexes in pupal development time; females developed slower (1 d) than males (Table 2.5).

Table 2.5: BSF pupal development (d) across trial (T1 = trial 1; T2 = trial = trail 2) and density. Pupae were kept in a walk-in growth chamber set at 30.2 ± 0.5°C with 60 ± 5.1% RH and 14:10 (L:D) conditions.

	Sex (Mean Days ± SEM)			
Density	M_{T1}	M_{T2}	F_{T1}	F_{T2}
500	14.2014 ± 0.1627A[1]a[2]	13.3417 ± 0.2471A	14.7726 ± 0.1184Aa	13.8584 ± 0.2176A
1000	13.7978 ± 0.0839ABa	13.1757 ± 0.1089B	13.6860 ± 0.0904Ca	13.6260 ± 0.1223A
1500	13.0906 ± 0.0723Bb	13.0753 ± 0.1390A	14.2219 ± 0.1295BCa	13.4206 ± 0.1863A
2000	13.7313 ± 0.1494ABb	12.9615 ± 0.0429B	14.3239 ± 0.1296Ba	13.6054 ± 0.0498A

[1, 2]Different capital letters within a column indicate significant ($p < 0.05$) differences across treatments, while different lowercase letters indicate significant ($p < 0.05$) differences between sexes of same treatment.

2.3.6 Adult emergence rates

No significant treatment by trial interaction ($F_{3,3} = 2.64$, $P = 0.0729$) or trial effect ($F_{1,1} = 0.0004$, $P = 0.9840$) was found for adult emergence (Table 2.6). Additionally, adult emergence did not differ across larval density ($F_{3,3} = 1.01$, $P = 0.4007$); emergence was > 90% for all larval densities.

Table 2.6: Percent emergence of adult BSF across densities. Adults were kept in a walk-in growth chamber set at 30.2 ± 0.5°C with 60 ± 5.1% RH and 14:10 (L:D) conditions. Adult emergence was calculated by averaging the ten percent of pupae collected that have emerged as adults within each trial.

Density	Adult Emergence (%) ± SEM
500	92.20 ± 1.1628A[1]
1000	90.08 ± 1.1628A
1500	92.56 ± 1.1628A
2000	92.65 ± 1.4241A

[1]Different letters within a column indicate significant ($p < 0.05$) differences.

2.3.7 Adult weight

No significant treatment by trial interaction ($F_{3,3}$ = 2.12, P = 0.1088) or trial effect ($F_{1,1}$ = 0.09, p = 0.7614) was found for adult weight. However, weights differed significantly across larval densities ($F_{7,59}$ = 13.22, P < 0.0001). The lowest density produced the heaviest adults (0.055 g), and the greatest density produced the smallest adults (0.039 g).

Significant differences ($F_{3,1}$ = 55.15, P < 0.0001) were found within each density when separated by sex (Table 2.7); females were 18.78% heavier than males.

Table 2.7: BSF adult weights (g) by sex for both trials. Adults were kept in a walk-in growth chamber set at 30.2 ± 0.5°C with 60 ± 5.1% RH and 14:10 (L:D) conditions.

	Sex (Mean ± SEM)	
Density	M	F
500	0.0512 ± 0.0015A[1] b[2]	0.0609 ± 0.0017Aa
1000	0.0407 ± 0.0011Bb	0.0478 ± 0.0013Ba
1500	0.0360 ± 0.0014Cb	0.0447 ± 0.0016Ba
2000	0.0360 ± 0.0020BCb	0.0445 ± 0.0021Ba

[1, 2]Different capital letters within a column indicate significant (p < 0.05) differences across treatments, while different lowercase letters indicate significant (p < 0.05) differences between sexes of same treatment.

2.3.8 Adult longevity

No significant interaction ($F_{3,3}$ = 0.92, P = 0.4330) was found between treatment and trial. However, a significant ($F_{1,1}$ = 5.12, P = 0.0278) trial effect was found. In trial one, no significant differences ($F_{3,31}$ = 1.99, P < 0.1371) in adult longevity were found across densities (Table 2.8). There was a significant difference ($F_{1,1}$ = 183.97, P < 0.0001) in adult longevity between males and females across all densities (Table 2.8) males lived longer (9.35 d) than females (7.52 d).

In trial two, no significant differences in adult longevity were found across all densities ($F_{3,3}$ = 0.56, P = 0.6420) (Table 2.8) with adults living approximately seven to eight days. However, a significant difference between sexes was found ($F_{1,1}$ = 221.01, P < 0.0001) (Table 2.8), and males lived longer (8.75 d) than females (6.85 d).

Table 2.8: BSF adult longevity (d) across trial (T1 = trial 1; T2 = trial = trail 2) and sex. Adults were kept in a walk-in growth chamber set at 30.2 ± 0.5°C with 60 ± 5.1% RH and 14:10 (L:D) conditions.

	Sex (Mean ± SEM)			
Density	M_{T1}	M_{T2}	F_{T1}	F_{T2}
500	8.6475 ± 0.1539B[1] b[2]	8.6550 ± 0.1865Ab	6.7075 ± 0.1539Ba	6.9875 ± 0.1865Aa
1000	9.3250 ± 0.2648ABb	8.6825 ± 0.1448Ab	7.7375 ± 0.2648Aa	6.7550 ± 0.1448Aa
1500	9.7425 ± 0.1308Ab	8.9375 ± 0.1678Ab	7.7905 ± 0.1308Aa	6.9100 ± 0.1678Aa
2000	9.7200 ± 0.1887Ab	8.7600 ± 0.1442Ab	7.7400 ± 0.1887Aa	6.7700 ± 0.1442Aa

[1, 2]Different capital letters within a column indicate significant (p < 0.05) differences across treatments, while different lowercase letters indicate significant (p < 0.05) differences between sexes of same treatment.

2.4 Discussion

The densities explored in this research as related to feed rate and resulting impacts on associated life-history traits of the BSF represented the largest scale examined to date with this species. As with previous studies, feed rate as related to larval density impacted many BSF life-history traits. Development time and weight of the BSF were significantly different between the lowest (500 larvae) and greatest (2000 larvae) densities across all life stages. Those reared at the lowest density (i.e., greatest feed rate) developed 18 d faster to the prepupal stage and were 24% larger across all life stages than those reared at the higher density (i.e., lowest feed rate). In general, adult longevity was not impacted. Such information is crucial for mass production of this species as slight

variations in larval density could impact output (e.g., waste conversion ratios and protein production).

Similar results were found in previous studies on BSF (Diener et al. 2009, Barragán-Fonseca et al. 2018). Diener et al. (2009) reported that BSF larvae provided a feed rate of 0.108 g/day exhibited greatest success in terms of production (e.g., prepupal weight and adult weight were greatest), similar to the feed rate (0.1 g/day) provided at the lowest density in the current study, which indicated a possible trend in feed rate per larva. Barragán-Fonseca et al. (2018) examined four densities with feeding rates ranging from 0.6 to 0.51 g/larva. As with Diener et al. (2009) and the current study, increased density impacted larval traits, such as larval weight and survivorship.

Besides competition for resources, other factors could be responsible for effects seen. For example, increased larval density could result in rapid shifts in environmental factors, such as pH and moisture, which could explain the impact on life-history traits. Paz et al. (2015) investigated the impact of larval density (2 larvae/cm^2 and 6 larvae/cm^2) on bioconversion of fruit and vegetable waste (fed at 0.06, 0.13 and 0.2 g/larva/d). They determined the mean pH was between 7 and 8 when larvae were fed at rates up to 0.06 g/larva/d, while those fed 0.2 g/larva/d (i.e., reduced competition) was lower (pH = 4 to 5). Such shifts could negatively impact corresponding BSF development. In fact, Ma et al. (2018) examined the impact of pH on the development of BSF larvae. They determined BSF larval development was the slowest (28 d to complete) at low pH (2.0) when compared to a diet with a pH of 8.0 (larval development of 21 d). Similar results were recorded for other life-history traits such as larval weight and survivorship. However, adult longevity was shortest (10 d) in a slightly basic diet rather than diet with a pH of 2.0 (15 d).

Moisture was also known to impact BSF development. Cheng et al. (2017) determined larval development time to the prepupal stage was 1-5 d faster when fed a diet with 80% rather than 70% moisture. They also found that final larval weights were ~0.01-0.04 g heavier when provided a diet with the greater moisture content. Similarly, Cammack and Tomberlin (2017) determined moisture content lower than 55% resulted in 100% larval mortality. However, even if initial moisture of the larval diet is optimal, shifts in moisture content can occur during larval feeding. Zheng et al. (2012) calculated the average moisture of the solid residual fraction of restaurant waste was reduced by 70-80% after digestion by BSF larvae. Initial moisture of chicken manure (77.18 ± 0.21%) was reduced by larval BSF to 55.27 ± 0.80%; and initial moisture content of dairy manure (75.46 ± 0.10%) was reduced by larval BSF to 66.08 ± 0.77% (Rehman et al. 2017). After digestion by the house fly, moisture reductions in diet residue (approximately 60-70%) were recorded (Barnard et al. 1998), and such shifts were positively correlated with larval density. However, such a shift in moisture had negative consequences on larval survivorship (97% at lowest density and 0.87% at the greatest density) and pupal weight (0.018 g/pupa at lowest and 0.005 g/pupa at greatest density). Like with pH, mass-production facilities should monitor initial moisture of the waste fed to BSF larvae, as well as monitor over time, to ensure optimal waste digestion and subsequent BSF production.

Results generated in the current study were for BSF larvae fed the Gainesville diet. Consequently, care should be taken when applying these results to BSF digestion of resources with varying nutrient quality, as responses could vary. For example, *Phormia regina* (Diptera: Calliphoridae) reared at densities of one and ten larvae/g on lamb liver and different protein to carbohydrate ratio (P:C) diets resulted in the shortest larval development time (~5 d). On a high protein diet (52:17), pupal weights were comparably high (~0.046 g) for both densities (Green et

al. 2003). In contrast, diets with almost equal P:C ratio (37:33) and lower protein (22:33) resulted in the slowest development time (~11 d) and smallest pupal weights (~0.027 g) in densities of one larva. Similar results were reported in Nguyen et al. (2013), where BSF fed on liver (P:C ratio at 39:2) reached prepupal stage faster (~19.17-32.17 d) than the BSF reared on fruits and vegetables (P:C ratio of (1.8:0.12) (development to prepupal stage estimated at ~21.67-40.33 d). Cammack and Tomberlin (2017) reared BSF larvae on artificial diets with varying P:C (7:35, 21:21, and 35:7) ratios and moisture contents (40, 55, or 70%). They determined BSF larvae developed slower on high-carb diets (0.375-6.875 d) when compared to those fed on high protein diets. Percent survivorship to pupal stage was higher (>50%) at diets with 70% moisture. More studies should examine the effects of macronutrient shifts in diet to physiological performances of both sexes of BSF.

Increased global demand for agricultural crops and livestock is a major issue due to the projected human population numbers to reach an estimated 9 billion by the year 2050. (Tilman et al. 2011). As previously stated, increased greenhouse gas emissions are partly associated with increased food and animal waste (Porter et al. 2016). The novel shift of mass production of insects to combat these issues can be the first step to tackle exponential population growth such as insects for human consumption (van Huis 2013), fish meal (Xiao et al. 2018), and waste management (Li et al. 2011, Banks et al. 2014). However, understanding the impacts of BSF larval density on their life history traits is crucial for efficiency of BSF mass production.

In summary, the impact of varying BSF larval densities on selected life-history traits was determined. The lowest larval density (500) resulted in heavier weights across all life history traits, shortest larval development time, and higher survivorship. Adult longevity was not impacted by these treatments. Because the study was conducted with four densities at increments of 500, data

generated could be applied more rapidly, rather than benchtop studies, by companies that mass produce the BSF. However, such companies should still exercise caution as these data are for a population in Texas, USA that originated in Georgia, USA. Thus, companies seeking to employ these results should conduct similar studies with their populations to ensure results are truly applicable.

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CHAPTER III

VALIDATION OF ACRYLIC PAINT AS A MARKING TECHNIQUE FOR EXAMINING MATING SUCCESS OF THE BLACK SOLDIER FLY *HERMETIA ILLUCENTS* (L.) (DIPTERA: STRATIOMYIDAE)

The black soldier fly, *Hermetia illucens*, (L.) (Diptera: Stratiomyidae) is mass produced worldwide for use in waste management and the production of an alternative protein for use as feed. However, few publications have explored its adult behavior, particularly mating success, as a means to optimize egg production in colony. In addition, there is limited knowledge of appropriate marking techniques to explore the mating behavior of this insect. The goal of this study was to validate water-based acrylic paint markers as a possible marking tool for behavioral studies with adult black soldier flies. Adult black soldier flies (<24-h-old) were marked with either green, gold, red, or white acrylic paint. Adult longevity, as well as the number of matings (mating success) were recorded for each treatment. Regardless of marking, 1) adult females lived approximately 7 d, while males lived 9 days, 2) mating frequency peaked two days after initiation of the experiment, 3) approximately 30% of adults across treatments were recorded mating, and 4) egg production and hatch rate were not affected. This marking technique did not affect the parameters measured, suggesting it is suitable for experiments requiring a reliable marking technique.

3.1 Introduction

Developing an appropriate technique for marking insects in research can be a challenge as materials used can negatively impact the insect. Walker and Wineriter (1981) suggested marking materials that are non-toxic, adhesive, quick-drying, lightweight, easy to apply, and easily visible

are ideal for conducting behavioral, or mark-recapture, studies with insects. Many mark-recapture studies for monitoring insect pest dispersal utilized fluorescent dusts (Hogsette 1984, Hagler and Jackson 2001, Coviella et al. 2006). Acrylic paints and genetic markers have also been widely used for monitoring dispersal of sterile insects released into the wild (Shearman et al. 2010, Gilchrist and Dominiak 2019). However, genetic markers, such as DNA microsatellites (Gilchrist and Dominiak 2019), can be cumbersome, time consuming, and expensive. Hence, cheaper and simpler method, such as acrylic paints, can be more practical.

The black soldier fly, *Hermetia illucens*, (L.) (Diptera: Stratiomyidae) is a cosmopolitant species, well known for its potential for sustainable waste management (Banks et al. 2014, Miranda et al. 2019) and alternative feed for livestock (Newton et al. 1977, St-Hilaire et al. 2007). With the increased interest in the black soldier fly, particularly with mass production, much research has focused on the optimization of egg production (Booth and Sheppard 1984, Tomberlin and Sheppard 2002, Zhang et al. 2010, Zheng et al. 2013, Park et al. 2016, Hoc et al. 2019). *H. illucens* displays lekking behavior (Tingle et al. 1975, Tomberlin and Sheppard 2001), whereby males battle other males on host-plant territories with the "winning" male obtaining mating privilege. Giunti et al. (2018) recently described male courtship behavior including male-male interactions and wing fanning. Mating frequency or choice, both of which can impact fertile egg production for use in industrialized settings, could be influenced by the lekking behavior. Consequently, understanding this behavior could influence the efficiency of any mass-rearing system. These studies could demand safe and efficient marking procedures that will not affect the fitness of the marked insects. However, a marking system has not been developed for use with the black soldier fly. This study evaluated the effect of acrylic paint as a marking technique for monitoring black soldier fly lekking behavior.

3.2 Methods

3.2.1 Acquisition of flies

Eggs collected from a black soldier fly colony maintained at the Forensic Laboratory for Investigative Entomological Sciences (F.L.I.E.S.) Facility at Texas A&M University (TAMU) in College Station, Texas, USA were used in these experiments. The colony was originally established in 2014 at the Coastal Plain Experiment Station, University of Georgia (Georgia, USA), from material collected at a poultry facility in Bacon Co., Georgia, USA, in 1998. At 0800 h, corrugated cardboard (8.8 x 5 x 1.2 cm) was placed on top of a screen lid attached to a (33.0 (L) x 21.6 (W) x 30.5 (D) cm) plastic box containing fifty black soldier fly neonates feeding in 100 g of the Gainesville house fly, diet (Hogsette 1992) with 70% water content to attract ovipositing individuals (Tomberlin et al. 2002). At 1700 h, resulting eggs were removed from the cardboard using a 21 cm VWR® disposable spatula, and placed in a 100 ml cup (Frontier Agricultural Services, Delaware, USA) with a lid and stored in a walk-in growth chamber set at 29.8 ± 0.8°C with 65.0 ± 5.3% RH and 14L:10D photo regime. Eggs were monitored every 12 h for eclosion, and approximately 10,000 resulting larvae were placed in a plastic 460 ml cup (Frontier Agricultural Services, Delaware, USA) containing 40 g of the Gainesville house fly diet with 70% water content, for 4 d (Sheppard et al. 2002).

3.2.2 Experimental design

Black soldier fly larvae (4 d) were divided into four replicates of 500 larvae per 532 ml cup (Solo Company, Illinois, USA), and fed 54 g of the Gainesville house fly diet (Hogsette 1992) every day until larvae reached 40% pupation (Jones and Tomberlin 2019). Cups containing larvae and diet were covered with white tulle fabric (Wal-Mart Stores Inc., Arkansas, USA), secured with a rubber

band, and placed individually in the center of a plastic pan (33.0 (L) x 21.6 (W) x 30.5 (D) cm). The plastic pans were then placed in a randomized complete block design on shelves in the walk-in growth chamber previously described. On the ninth day, the 532 ml cups were then emptied into the (33.0 (L) x 21.6 (W) x 30.5 (D) cm) plastic containers, which were also covered with tulle fabric secured with elastic cord, and larvae were fed as described above. Prepupae were placed in separate (33.0 (L) x 21.6 (W) x 30.5 (D) cm) plastic containers covered with tulle fabric. Two hundred emergent flies (<24-h-old) were sexed and 100 were marked on their thorax with one of the randomly assigned colors: red, gold, green, or white, using 3 mm tip Garde'n'Craft® Fine Point Markers (Uchida of America Corp, California, USA). Emergent black soldier flies are extremely docile and soft-bodied; therefore, handling time was less than a minute. Flies were held between two fingers with elbows resting on hard surface for balance. Unmarked adults were held as previously stated, but not marked. Males and females were placed in separate 30.0 x 30.0 x 30.0 cm cages (BioQuip Products, California, USA) until experiment use. Black soldier fly adult males emerge 2 d before females (Tomberlin and Sheppard 2002). Therefore, ages of males and females were mixed in order to reach equal sex ratio. Flies were provided with water *ad libitum*, as adults do not need to feed.

3.2.3 Impact of marking flies on mating

The study was conducted from August 2017 to February 2018. Approximately 200 black soldier fly adults of 1:1 sex ratio were released in each of four 84 (L) x 84 (W) x 132 (H) cm cages assigned to each color treatment (Insect-A-Hide™ pop-up shelter, Lee Valley Tools, Ltd., New York, USA) inside of a greenhouse. The number of mating pairs for marked and unmarked individuals was recorded for ten minutes every hour from 0800 to 1700 h over a 4-d period, which is the window

for such behavior to occur (Tomberlin and Sheppard 2001). Cages were misted with water (10 ml per cage) every two hours during each observation period to enhance the longevity of the adult flies (Tomberlin et al. 2002). Relative humidity, temperature, and light intensity were recorded using a HOBO® data logger model U12-012 (Onset Computer, Co. Massachusetts, USA). Temperature inside the cages was also recorded using Acu-Rite® Thermometers (Lake Geneva, Wisconsin, USA). Two trials were conducted for the following combinations in cages, 1) marked males and unmarked females, 2) marked females and unmarked males, or 3) both marked and unmarked males and females. Color assignments were randomized during each observation period. For a trial, a cage containing flies receiving an assigned color marking was considered a replicate (n = 4).

3.2.4 Impact of marking flies on oviposition and hatch rate

The study was conducted from March 2018 to May 2018. Methods utilized in marking flies to monitor mating frequency were applied as described above. Two treatments were examined in this study. To account for limited number of cages, adult males and females within a cage were marked with either green and white or red and gold on their thorax or remained unmarked in control cages. For each color combination, two replicates of each treatment and the control were used for two trials.

Methods for harvesting eggs were based on those previously described by Tomberlin and Sheppard (2002). Following the initiation of the experiment, eggs were collected daily on days three through five which coincided with peak oviposition behavior by the black soldier fly (Tomberlin and Sheppard 2002). A corrugated cardboard block (5 (L) x 2.54 (W) x 1.27 (H) cm) was placed on top of a 400 ml plastic box containing 300 g Gainesville diet with 70% water

content, which was placed in the center of each cage. The corrugated cardboard block was replaced daily at 0700 h. Egg clutches present in the cardboard were removed using a 21 cm VWR® disposable spatula and individually weighed on an Ohaus Scout® Pro Balance Scale (Ohaus Corporation, New Jersey, USA). In order to estimate the number of eggs gravimetrically (Booth and Sheppard, 1984), 200 eggs were counted to determine a standard weight and calculated with the total number of eggs laid during the experiment. Resulting eggs were kept in a 59 ml cup and placed in a walk-in growth chamber as previously described. Egg production by day was calculated to give total number of eggs produced. Hatch rate for each treatment was calculated by dividing the number of viable larvae hatched after 4 d from oviposition from the number of eggs produced.

3.2.5 Longevity study

The impact of this marking system on adult longevity was determined. Adults were obtained using the method described above. Methods for producing adults previously described were used. Fifteen of each initial emergent male and female flies (<24-h-old) were assigned to each color treatment. The same number of adults remained unmarked and served as controls. Adults were placed individually in 59 ml cups, capped with a breathable lid, labeled, and placed in the walk-in growth chamber previously described. They were provided water *ad libitum* via a cotton ball placed through the lid (Tomberlin et al. 2002), and mortality was recorded daily.

3.2.6 Statistics

All statistical analyses were performed using R version 3.5.1. A Shapiro-Wilk test for normal distribution, and Levene Test for homogeneity of variance were implemented to test for normality assumptions. A paired t-test was used to compare number of mating pairs by marked and unmarked

males or females. A repeated measures one-way analysis of variance (ANOVA) was used to compare number of mating pairs by marked and unmarked males and females. A two-way analysis of variance (ANOVA) was implemented to determine if color and day did not influence mate choice of marked and unmarked individuals. Oviposition data, including oviposition over time, did not meet normality assumptions, therefore a Kruskal-Wallis test was used to compare the number of eggs produced by marked females with unmarked ones. Pearson's product-moment correlation analysis was used to determine level of correlation between mating frequency and egg production with temperature, relative humidity (RH), and light intensity. For the longevity study, parametric assumptions were met; therefore, an ANOVA was used to test for differences between colors and adult longevity. Alpha for all analyses was set at 0.05.

3.3 Results

3.3.1 Impact on mating: marked males only

The temperature range inside cages was 20.9-32.2°C (mean 26.6°C ± 3.7). Greenhouse temperatures ranged from 19.8-35.0°C (mean 25.1°C ± 4.8); and relative humidity (RH) from 47.7-60.6% (mean 52.5% ± 3.2). Mating frequency was not correlated to temperature ($r = 0.1012$, $df = 58$, $p = 0.4416$) or RH ($r = 0.0291$, $df = 58$, $p = 0.8251$), but it was positively correlated with light intensity ($r = 0.3200$, $df = 58$, $p = 0.0126$). Overall, there was no significant difference in mate choice by marked and unmarked males ($t = 0.84615$, $df = 1$, $p = 0.5529$) (Table 3.1). Number of mating pairs did not differ per day between marked and unmarked males ($F = 3.614$, $df = 1$, $p = 0.1300$) (Table 3.1). Additionally, color did not affect mating frequency between marked and unmarked males ($F = 0.0380$, $df = 3$, $p = 0.9890$) (Table 3.1), and there was no significant interaction of color*day of the experiment either ($F = 0.289$, $df = 3$, $p = 0.8320$) (Table 3.1).

Table 3.1: Total number of black soldier fly mating pairs ± SD (n = 4; N = 200; 100 ♂ 100 ♀) observed in all experiments where males were marked with a single color on their thorax. Adults were observed in 84 (L) x 84 (W) x 132 (H) cm cages in a greenhouse maintained at 25.1°C ± 4.8 and 52.5% RH ± 3.2.

	MM[1]+UF[2] ± SD				UM[3]+UF[4] ± SD			
	Green[5]	Gold	Red	White	Green	Gold	Red	White
Day								
1	2.00 ± 0.50	3.00 ± 0.50	5.00 ± 0.50	3.00 ± 0.50	3.00 ± 0.06	2.00 ± 0.77	6.00 ± 0.50	4.00 ± 0.50
2	5.00 ± 0.50	7.00 ± 0.50	3.00 ± 2.00	6.00 ± 0.50	4.00 ± 0.77	6.00 ± 0.67	3.00 ± 0.50	5.00 ± 1.00
3	1.00 ± 0.50	1.00 ± 0.67	0.00 ± 0.50	1.00 ± 0.67	1.00 ± 1.27	1.00 ± 0.50	1.00 ± 0.50	1.00 ± 0.50
4[6]	--	--	--	--	--	--	--	--
Total	8.00 ± 1.50	11.00 ± 1.67	8.00 ± 2.50	10.00 ± 1.67	8.00 ± 2.10	9.00 ± 1.94	10.00 ± 1.50	10.00 ± 2.00

[1]MM = marked males, [2]UF = unmarked females, [3]UM = Unmarked males, [4]UF = unmarked females.
[5]Color treatments
[6]Day 4 showed no mating observations.

3.3.2 Impact on mating: marked females only

The temperature range inside cages was 22.2-36.1°C (mean 29.2°C ± 4.1). Greenhouse temperatures ranged from 19.2-34.8°C (mean 25.8°C ± 4.5), while RH ranged from 42.6-61.8 % (mean 50.7% ± 5.4). As with marked males, analyses indicated no correlation between temperature ($r = -0.0380$, $df = 58$, $p = 0.7731$) or RH ($r = -0.0552$, $df = 58$, $p = 0.6751$) and mating frequency whereas it was positively correlated with light intensity ($r = 0.2591$, $df = 58$, $p = 0.0458$). No difference in mate choice of marked and unmarked females ($t = 0.53612$, $df = 1$, $p = 0.6867$) (Table 3.2) was observed. Number of mating pairs of marked or unmarked females did not differ per day of the experiment either ($F = 1.8820$, $df = 1$, $p = 0.242$) (Table 3.2). Additionally, color did not affect mating frequency ($F = 0.0780$, $df = 3$, $p = 0.9680$), and there was no significant interaction of color*day of the experiment ($F = 0.0780$, $df = 3$, $p = 0.9680$) (Table 3.2).

Table 3.2: Total number of black soldier fly mating pairs ± SD (n = 4; N = 200; 100 ♂ 100 ♀) observed in all experiments where females were marked with a single color on their thorax. Adults were observed in 84 (L) x 84 (W) x 132 (H) cm cages in a greenhouse maintained at 25.8°C ± 4.5 and 50.7% RH ± 5.4.

	MM[1]+UF[2] ± SD				UM[3]+UF[4] ± SD			
	Green[5]	Gold	Red	White	Green	Gold	Red	White
Day								
1	2.00 ± 0.50	3.00 ± 0.50	5.00 ± 0.50	3.00 ± 0.50	3.00 ± 2.06	2.00 ± 0.77	6.00 ± 0.50	4.00 ± 0.50
2	5.00 ± 0.50	7.00 ± 0.50	3.00 ± 2.00	6.00 ± 0.50	4.00 ± 1.77	6.00 ± 0.67	3.00 ± 0.50	5.00 ± 1.00
3	1.00 ± 0.50	1.00 ± 0.67	0 ± 0.50	1.00 ± 0.67	1.00 ± 2.27	1.00 ± 0.50	1.00 ± 0.50	1.00 ± 0.50
4[6]	--	--	--	--	--	--	--	--
Total	8	11	8	10	8	9	10	10

[1]MM = marked males, [2]UF = unmarked females, [3]UM = Unmarked males, [4]UF = unmarked females.
[5]Color treatments
[6]Day 4 showed no mating observations.

3.3.3 Impact on mating: marked males and females combined

The temperature range inside cages was 20.0-31.6°C (mean 24.3°C ± 2.9). Greenhouse temperatures ranged from 17.7-29.4°C (mean 23.4°C ± 3.1); and RH from 40.7-64.0% (average 52.4% ± 8.3). As with the previous tests, there was, no significant correlation between mating frequency and temperature ($r = 0.0683$, $df = 58$, $p = 0.6041$) or RH ($r = 0.1325$, $df = 58$, $p = 0.3125$), but it was positively correlated with light intensity ($r = 0.3040$, $df = 58$, $p = 0.0182$). No significant difference ($F = 0.8663$, $df = 3$, $p = 0.4682$) of mate choice was found for marked or unmarked males and females (Table 3.3). Number of marked and unmarked mating pairs of males and females did not differ for experiment day ($F = 1.2790$, $df = 1$, $p = 0.3210$) (Table 3.3). Marker colors had no significant impact on mate choice by either sex ($F = 0.0250$, $df = 3$, $p = 0.9940$) (Table 3.3), and there was no significant interaction of color*day of the experiment ($F = 0.0430$, $df = 3$, $p = 0.9870$) (Table 3.3).

Table 3.3: Total number of black soldier fly mating pairs ± SD (n = 4; N = 200; 100 ♂ 100 ♀) observed in all experiments where males and females were marked with a single color on their thorax. Adults were observed in 84 (L) x 84 (W) x 132 (H) cm cages in a greenhouse maintained at 28.4°C ± 5.3 and 60.1% RH ± 5.5.

	MM[1]+MF[2] ± SD				MM+UF[3] ± SD				MF+ UM[4] ± SD				UF+UM ± SD			
	Green[5]	Gold	Red	White	Green	Gold	Red	White	Green	Gold	Red	White	Green	Gold	Red	White
Day																
1	2.00 ± 0.67	2.00 ± 0.95	2.00 ± 0.67	4.00 ± 0.81	2.00 ± 0.67	3.00 ± 0.97	3.00 ± 1.28	4.00 ± 0.93	3.00 ± 1.01	2.00 ± 0.50	4.00 ± 1.21	3.00 ± 0.93	5.00 ± 0.81	4.00 ± 1.17	3.00 ± 0.77	3.00 ± 1.13
2	5.00 ± 0.95	5.00 ± 0.57	6.00 ± 0.67	5.00 ± 1.07	3.00 ± 1.13	4.00 ± 0.77	4.00 ± 0.67	5.00 ± 0.50	5.00 ± 1.03	5.00 ± 1.03	3.00 ± 0.93	6.00 ± 0.81	4.00 ± 1.13	4.00 ± 0.93	5.00 ± 0.95	6.00 ± 1.07
3	1.00 ± 0.95	2.00 ± 0.57	1.00 ± 0.81	0.00 ± 0.50	3.00 ± 0.82	2.00 ± 0.50	1.00 ± 0.67	1.00 ± 0.77	1.00 ± 0.50	1.00 ± 0.23	2.00 ± 0.67	1.00 ± 0.50	2.00 ± 0.50	1.00 ± 0.50	2.00 ± 0.23	2.00 ± 0.67
4[6]	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Total	8.00 ± 2.57	9.00 ± 2.09	9.00 ± 2.15	9.00 ± 2.38	8.00 ± 2.62	9.00 ± 2.24	8.00 ± 2.62	10.00 ± 2.20	9.00 ± 2.54	8.00 ± 1.76	9.00 ± 2.81	10.00 ± 2.24	11.00 ± 2.44	9.00 ± 2.60	10.00 ± 1.95	11.00 ± 2.87

[1]MM = marked males, [2]MF = marked females, [3]UF = unmarked females, and [4]UM = unmarked males
[5]Color treatments
[6]Day 4 showed no mating observations.

3.3.4 Impact of marking on egg production

The temperature inside cages was 28.6-31.8°C (mean 29.7 ± 3.3°C). Greenhouse temperatures ranged from 26.9-30.4°C (mean 28.4 ± 5.3°C); and relative humidity (RH) from 56.7-69.6% (mean 60.1 ± 5.5%). No significant trial effect (H = 0.2273, df = 2, p = 0.8925), or trial by treatment effect (H = 6.561, df = 3, p = 0.0872) was found on number of eggs. Neither color (H = 5.303, df = 2, p = 0.0706) nor marking affected (H = 0.1448, df = 1, p = 0.7036) number of eggs (Table 3.4) or hatch rate (H = 0.0123, df = 1, p-value = 0.9115) (Table 3.4). In addition, egg production by day for marked and unmarked cages showed no significant differences (H = 0.9631, df = 4, p = 0.9153) (Table 3.4). Correlation analyses indicated positive correlation with temperature (r = 0.3698, df = 29, p = 0.0463) and RH (r = 0.3758, df = 29, p = 0.0434) on egg production, but not with light intensity (r = -0.1289, df = 29, p = 0.5031).

Table 3.4: Total number of eggs ± SD, and hatch ± SD (n = 4; N = 200; 100 ♂ 100 ♀) by marked and unmarked adults observed in 84 (L) x 84 (W) x 132 (H) cm cages in a greenhouse maintained at 28.4°C ± 5.3 and 60.1% RH ± 5.5.

	Experiment One[1]		Experiment Two[2]	
	Marked	Unmarked	Marked	Unmarked
Day				
1	4457.00 ± 589.14	5691.00 ± 667.75	3211.00 ± 791.14	3515.00 ± 712.44
2	9984.00 ± 1669.56	11934.00 ± 1354.79	5784.00 ± 2416.21	4748.00 ± 1810.54
3	2516.00 ± 658.94	1532.00 ± 323.11	3401.00 ± 716.56	1708.00 ± 946.71
4	--	--	--	--
Total Number of Eggs	16957.00 ± 1597.14	19157.00 ± 2845.45	12395.00 ± 1885.18	9971.00 ± 1298.52
Total Hatch Rate (%)	62.00 ± 5.97	69.00 ± 8.67	71.00 ± 6.27	64.00 ± 4.25

[1]Color combinations of green and white for marked adults.
[2]Color combinations of red and gold for marked adults.

3.3.5 Impact of marking on adult longevity

No significant trial effect ($F_{1,1}$ = 0.48, p = 0.4891), or trial by treatment effect ($F_{3,3}$ = 0.96, p = 0.4105) was found on adult longevity. Neither color ($F_{3,3}$ = 0.91, p = 0.4345) nor marking affected ($F_{1,1}$ = 0.17, p = 0.6787) adult longevity (Table 3.5). Sex did not significantly ($F_{3,3}$ = 1.41, p = 0.2407) impact longevity either.

Table 3.5: Mean adult longevity of marked and unmarked black soldier fly adults ± SE (n = 4). Larvae were reared in a walk-in growth chamber set at 29.8°C ± 0.8 with 65.0% RH ± 5.3 and 14:10 (L:D) conditions.

	Sex (Mean ± SE)			
	[1]MF	[2]UF	[3]MM	[4]UM
Color				
Green	7.23 ± 0.34[5]A[6]a	7.26 ± 0.34Aa	8.66 ± 0.34Aa	8.96 ± 0.34Aa
Gold	7.13 ± 0.34Aa	7.30 ± 0.34Aa	8.86 ± 0.34Aa	8.50 ± 0.34Aa
Red	7.10 ± 0.31Aa	7.20 ± 0.31Aa	9.03 ± 0.31Aa	9.00 ± 0.31Aa
White	7.53 ± 0.30Aa	7.23 ± 0.30Aa	9.56 ± 0.30Aa	9.06 ± 0.30Aa

[1]MF = marked females, [2]UF = unmarked females, [3]MM = Marked males, [4]UM = unmarked males.
[5]Capital letters within a column indicate no significant (p > 0.05) differences across color treatments within a sex; [6]lowercase letters indicate no significant (p > 0.05) differences between sexes within the same color treatment.

3.4 Discussion

Using the acrylic paints as a marking system had no impact on the mating success, egg production, hatch rate, or adult longevity of the black soldier fly (Table 3.4). Color used to mark adults did not affect mate choice either. Number of successful mating pairs by marked and unmarked adults did not differ over time. In spite of the frequent misting process in the black soldier fly cages, (Tomberlin et al. 2002), colors remained unaffected throughout the study.

The acrylic paints have not been useful for other insects due to negatively impacted marked individuals. In some instances, the meticulous application of paints to each individual can be time

consuming (Walker and Wineriter 1981). In other cases, these paints impacted insect mobility, and only lasted for 48 h (Mattioli and Walsh 2008). None of these issues were observed in the current study.

In addition, and while not a specific aim of this project, the impact of temperature and humidity were investigated *a posteriori* given the data were already recorded. We determined a high degree of variation in light intensity (200-42,000 lux) and relative humidity (40.7-64.0%) were experienced in the greenhouse. Fortunately, the acrylic paints were able to withstand such variation and were detectable on the flies throughout the experiments. As far as mating frequency, it was only significantly correlated to light intensity. This does not come as a surprise as such factors are known to play a role in such behaviors (Tomberlin and Sheppard 2002). In addition, temperature and humidity were significantly correlated with egg production, as found in similar work with (Holmes et al. 2012). Such information should be considered when establishing a mass-production site in order to stabilize production. Furthermore, future research is sorely needed to determine appropriate ranges for each of these factors as a means to optimize production in such industrialized facilities.

This study determined acrylic paint marking of adult black soldier flies did not affect their mating success, egg production, hatch rate, or adult longevity. Therefore, we recommend more exploration into the use of acrylic paint markers for black soldier fly mating studies in controlled environments. The use of acrylic paint markers can also be an inexpensive, effective method in mark-recapture studies.

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CHAPTER IV

EFFECTS OF ADULT BODY SIZE ON MATING SUCCESS OF THE BLACK SOLDIER FLY, *HERMETIA ILLUCENS* (L.) (DIPTERA: STRATIOMYIDAE)

Body size is a recognized factor impacting mating success of a number of insect species. The black soldier fly, *Hermetia illucens* (L.) (Diptera: Stratiomyidae), which is mass produced to convert organic waste to protein, exhibits a lekking behavior necessary for mating. However, it is not known if adult body size impacts mating success and subsequent fertile egg production. In this study, larvae were raised at two densities to produce two size classes of adults (i.e., large and small). Hourly mating observations were recorded in the following studies: 1) Homogenous populations of large or small adults; 2) 50% Heterogenous populations (equal number of large males with small females and vice versa); 3) and 25% heterogenous (i.e., equal number of large and small adults for both sexes). Adult weight, morphometrics of resulting adults, total number of mating pairs and failed mating attempts, multiple matings, as well as eggs produced and associated hatch rate were recorded for each experiment. Morphometrics and weights in large adults were 21% and 50% greater than small adult males and females, respectively. Homogenous populations (i.e., large or small) showed no significant differences across other variables measured. However, when populations of different sized adults were mixed equally based on sex (i.e., 50% heterogenous populations), mating success increased 50% to 100% for small males with large females and large males with small females, respectively. Total number of multiple matings increased two to three times. Egg production decreased 15-20% and hatch rate declined approximately 10%. In the 25% heterogenous populations, data were more complex. Number of successful mating pairs across male size was 50% greater than in the homogenous populations.

Number of failed mating attempts was also two to three times greater. Multiple matings overall were low (10%) for the homogenous and heterogenous populations. Large males demonstrated two times more aggression in general than small males in the heterogenous than the homogenous population. Approximately 30 to 400% more eggs were produced in the 25% heterogenous population than large or small homogenous populations, respectively, while hatch rate remained non-significant with an average of 70%. However, the variability in egg hatch was forty times greater than the large homogenous and 40% greater than the small homogenous. While increased egg production is desired, high variability in egg hatch impedes fertile egg production and predictability at an industrial scale.

4.1 Introduction

Body size, especially in males, is a key factor regulating mating success. For example, male *Cotesia urabae* (Austin and Allen) (Hymenoptera: Braconidae) with larger wings are typically favored by females (Avila et al., 2017). Secondarily, in some instances, the advantage of males having a larger body is increased mating success, such as recorded for mayfly (Ephemeroptera: Heptageniidae) mating-swarms (Flecker et al., 1988). In both instances, larger males have a greater likelihood of passing their genes to the next generation.

Body size of resulting adult insects is often a result of larval feeding (i.e., competition, resource available or quality). The link between larval competition and reproductive success has been thoroughly investigated with insects that feed as adults (Fricke et al., 2015; Hooper et al., 2017). For species that do not feed as adults, which is important for the current study (see discussion on black soldier fly below), such as male mayflies *Baetis bicaudatus* (Dodds) (Ephemeroptera: Baetidae), selective pressures have resulted in extended development time and

increased adult size, that offers a competitive advantage for a mate (Peckarsky et al., 2002). However, increased risk of predation is a trade-off with taking longer to develop (Peckarsky et al., 2001). Furthermore, in instances where males aggregate, or lek, size could be nullified, or become a disadvantage, if not capable of competing for mates.

Black soldier fly larvae demonstrate aggregative feeding at levels where the species historically was considered a pest in the livestock industry (Sheppard et al., 1994). Black soldier fly larvae have been found to breed in densities that yield up to 53 ton of prepupae over a five-month period (Sheppard et al., 2000), and black soldier fly adults lay up to a thousand eggs/adult on poultry manure patches (Axtell and Arends, 1990). However, that recognition as a pest shifted after associated benefits of the species in sustainable agriculture were determined.

Black soldier fly larvae have been documented as consumers of organic materials ranging from food waste (Cheng et al., 2015) to livestock manure (Beskin et al., 2018; Miranda et al., 2019). Due to their ability to convert a variety of wastes into biomass, the black soldier fly has been industrialized globally as a feed (van Huis, 2013). In fact, the larvae contain high protein (40%) and fat content (30%) (D. C. Sheppard et al., 1994) and has currently been approved in the United States and European Union as alternative feed for poultry and aquaculture.

Black soldier flies are highly plastic with regards to size (Jones and Tomberlin, 2019). A number of studies have investigated the impact of different feeding rates of various black soldier fly larval densities on their development time and resulting body sizes (Barragan-Fonseca et al., 2009; Myers et al., 2008; Parra Paz et al., 2015). Most reported that high larval densities with limited food resources resulted in smaller larvae and prepupae, compared to experimental treatments with low density and optimal food availability. Jones and Tomberlin (2019) extended

these efforts and demonstrated by manipulating larval density and feed rate, adult size could vary by as much as 26%.

Little information is known about adult biology of the black soldier fly (Tingle et al., 1975; Tomberlin and Sheppard, 2001). Adult black soldier flies appear to rely on energy reserves obtained during larval development to aggregate, secure mates, and produce offspring (Sheppard et al., 2002; Tomberlin and Sheppard, 2002). In fact, adults do not need to feed and only need water to survive (Tomberlin et al., 2002). Females typically mate once and lay a single clutch after mating and then die (Tomberlin and Sheppard, 2002); however, there is evidence that multiple matings can occur (Giunti et al., 2018).

The impact of adult size on mating success by the black soldier fly has not been investigated. For *Hermetia comstocki*, (Williston) (Diptera: Stratiomyidae), which also leks, larger males had greater mating success than smaller adult males (Alcock, 1990). However, potential sexual conflict within *H. illucens* could occur during mating (Giunti et al., 2018). Males are known to rapidly beat their wings and tap the female abdomen as they attempted to copulate. Male size could be linked with wing-beating frequency, duration, and successful copulation. However, additional studies are needed to test this hypothesis.

The purpose of this study was to investigate the impact of black soldier fly adult size on mating success. Data collected included number of total mating pairs observed in a population, frequency of mating, multiple matings, failed matings, male aggression, number of eggs deposited, and hatch rate. The following three comparisons were accomplished: 1) homogenous populations (i.e., controls) of large or small adults; 2) heterogenous populations of males from the "large" population with females from the "small" population, and the inverse; 3) and another heterogenous

study similar to the second objective, but 25% of each sex from the "large" and "small" populations comprising the fly population in a cage.

4.2 Methods

4.2.1 Acquisition of flies

Eggs were collected from a black soldier fly colony maintained at the Forensic Laboratory for Investigative Entomological Sciences (F.L.I.E.S.) Facility at Texas A&M University (TAMU) in Texas, USA. The colony was established in 2014 from eggs from a laboratory colony established at the Coastal Plain Experiment Station, University of Georgia, Georgia, USA, which originated from material collected at a poultry facility in Bacon Co., Georgia, USA, in 1998. At 0800 h, corrugated cardboard (8.8 (L) x 5.0 (W) x 1.2 (H) cm) was placed on top of a screen lid attached to a plastic box (33.0 (L) x 21.6 (W) x 30.5 (H) cm) containing 100 g of the Gainesville house fly, *Musca domestica*, L. (Diptera: Muscidae) diet (Hogsette 1992) at 70% moisture to attract ovipositing individuals (Tomberlin et al., 2002). After 8 h, resulting eggs were removed from the cardboard using a 21 cm VWR® Disposable Spatula and placed in a 100 ml cup (Frontier Agricultural Services, Delaware, USA) with a lid and stored in a walk-in growth chamber set at 29.5 ± 0.7°C with 66.0 ± 5.6% RH and 14:10 L:D photo regime. Eggs were monitored every 12 h for eclosion, and larvae were placed in a plastic 460 ml cup (Frontier Agricultural Services, Delaware, USA) and fed 40 g (at 70% moisture) of the Gainesville house fly diet for four days (Myers et al., 2008). Egg collection for producing small and large adults (defined below) were staggered based on timeframe for each population density to reach similar adult emergence time (Jones and Tomberlin, 2019).

4.2.2 Experiment design

Methods were adapted from Jones and Tomberlin (2019). Newly hatched black soldier fly larvae were placed in 532 ml cups (Solo Company, Illinois, USA) containing 40 g of the Gainesville house fly diet. After four days of feeding, the larvae were hand counted into six replicates of 500 and 2000 larvae/532 ml cups placed in the center of a plastic 4 L pan (33.0 (L) x 21.6 (W) x 30.5 (H) cm), totaling twelve replicates per trial. The cups were covered with white tulle fabric (Wal-Mart Stores Inc., Arkansas, USA) and secured by a rubber band. The plastic pans were then placed in a randomized block design on (183.0 (L) x 95.1 (W) x 52.3 (D) cm) shelves in the walk-in growth chamber previously described. Larvae in all replicates of each treatment were fed 54 g Gainesville house fly diet at 70% moisture daily until 40% of the larvae in the pan reached the prepupal stage. Prepupae were placed in a separate (33.0 (L) x 21.6 (W) x 30.5 (H) cm) plastic containers covered with tulle fabric. Emergent flies were weighed, sexed, and marked using a previously validated technique for black soldier fly (Jones and Tomberlin, 2020 *in review)*. Black soldier fly adult treatments (<24 h) were randomly assigned one of four color acrylic paint (e.g., green, red, gold, and white) based on their respective rearing density (i.e., large or small) and sex. For example, large males were marked with green, small females were marked with red, large females were marked with gold, and small males were marked with white. Colors were rotated during each trial, and adults were marked with 3 mm tipped Garde'n'Craft® Fine Point Terra-Cotta Markers (Uchida of America Corp, California, USA) on their thorax. Adult males typically emerge two days before females (Sheppard et al., 2002); therefore, to reach equal sex ratio, the oldest age of flies at initiation of observation study were five days old. For each replicate, males and females were kept in separate (30 (L) x 30 (W) x 30 (H) cm) plastic cages (Bioquip Products, California, USA). Flies were provided with water *ad libitum* before initiation of mating observations.

4.2.3 Mating observations

A combination of 100 males and 100 females was released to their respective 83.8 (L) x 83.8 (W) x 128.3 (H) cm cage (Insect-A-Hide™ pop-up shelter, Lee Valley Tools, Ltd., New York, USA) in the following consecutive experiments inside a greenhouse; 1) separate cages for large and small adults (designated homogenous populations), 2) separate heterogenous populations of large males and small females as well as small males with large females at equal numbers (designated as 50% population), and 3) and another separate heterogenous study similar to the second objective, but each treatment contained 25% of each size and sex (designated as 25% population). Cages were misted with water (10 ml per cage) every two hours during daylight hours. Mating observations of flies in each cage were made for ten minutes hourly from 0800 h to 1700 h for four days as this is the primary period of mating and oviposition activity (Tomberlin and Sheppard, 2002). Light intensity, humidity, and temperature were recorded daily during observation using a HOBO® data logger model U12-012 (Onset Computer Co., Massachusetts, USA). Temperature inside the cages were also recorded using Acu-Rite® Thermometers (Lake Geneva, Wisconsin, USA). Mating pairs were marked on their thorax using the marking technique previously discussed (Jones and Tomberlin, 2020 *in review*). Color assignment sequence with regards to mating number observed was randomly determined prior to each trial. For example, white would indicate a first mating, red the second mating, gold the third mating, and so on. This approach allowed the total number of matings observed for a given individual to be recorded. Mating successes, failures, and multiple matings were documented for flies in each treatment. Male-to-male aggression was recorded from the 25% heterogenous population treatment only to determine which different sized males within a heterogenous populations display more aggressive behavior. Each experiment was repeated three times.

4.2.4 Oviposition success and hatch rate

As previously described, eggs were collected by placing corrugated cardboard (5.0 (L) x 2.5 (W) x 1.2 (H) cm) on top of a 400 ml container that held 300 g (70% moisture) of the Gainesville house fly covered with a tulle cloth and secured by a rubber band in each cage. Females oviposit after two days of mating (Tomberlin and Sheppard, 2002), therefore, the container was placed in the center of each cage at 0800 h from days 3 to 5 of the experiment and changed daily to prevent mold formation. The container was removed from each cage at 0700 h the following day. Egg clutches were removed from the cardboard using a 21 cm VWR® Disposable Spatula and individually weighed on an Ohaus Scout® Pro Balance Scale (Ohaus Corporation, New Jersey, USA) to estimate the number of eggs gravimetrically (Booth and Sheppard, 1984). Individual clutches were placed in 100 ml cups covered with a breathable lid, labeled, and placed in the walk-in growth chamber previously described and allowed to hatch. Total number of eggs were determined for each treatment by counting and weighing eggs to retrieve gravimetric estimates. Total larval hatch rate for each treatment was determined by counting and weighing larvae to retrieve gravimetric estimates on number of larvae. Hatch rate was calculated by dividing the number of viable larvae from the number of eggs produced.

4.2.5 Morphometrics

A sample of ten male and female adults (<24 h) from each size and replicate were individually placed in separate 25 ml vials containing 75% ethanol. Morphometrics of black soldier fly adults were measured under a dissecting scope (Meiji Techno Company, Saitama, Japan) using Lumenera Infinity Analyze and Capture software version 6.2.3 (Teledyne Lumenera Imaging Group, Ontario, Canada). Magnifications were set at 70x for the following adult black soldier fly morphometrics:

wing length, abdominal length, and leg length. Magnifications were set at 100x for the following adult black soldier fly morphometrics: head width, antennal length, eye length, and external genitalia length. Antennae were measured from the scape to the eight flagellomere. Head width, eye length, and eye width measurements were taken dorsoventrally. Wing measurements were estimated using wing centroids (Klingenberg, McIntyre, & Zaklan, 1998). Genitalia were measured using the lengths of the aedeagus (male), and tubular oviduct (female).

4.2.6 Statistical analysis

All statistical analyses were performed using R® software version 3.5.3. Normality assumptions were also determined beforehand by using Shapiro-Wilk normality test for possible normal data distribution, and a Lavene's Test was used for possible homogeneity of variance. A t-test was used to compare homogenous populations of large and small adults by number of matings, number of eggs, number of larvae, hatch rate. An analysis of variance (ANOVA) was implemented to compare number of matings (successful, failed, and multiple), number of eggs, number of larvae, and hatch rate across treatments by day. A Tukey Honest Significant Differences (HSD) analysis followed significant means ($P < 0.05$). Kruskal-Wallis test was used on data that did not meet normality assumptions. A Dunn's post-hoc test followed non-parametric test significance ($P < 0.05$) to separate means. A MANOVA was utilized to analyze the morphometric data, followed by an ANOVA for specific traits determined to be significant. A Tukey HSD analysis followed significant means ($P < 0.05$). A likelihood ratio analysis was used to analyze the odds of large males mating with small females, and small males mating with large females.

4.3 Results

4.3.1 Adult size

Adult weights differed ($F_{3,20} = 88.0092$, $P < 0.0001$) between sexes in all three experiments (Figure 4.1). Mean weights for each sex and size are described: large females (0.05 ± 0.01 g), large males (0.04 ± 0.01 g), small females (0.03 ± 0.01 g), and small males (0.02 ± 0.01 g). Thus, adults in the large population were twice the size of their respective sex in the small population.

Figure 4.1: Mean weight (g) ± SD (n = 4) of black soldier fly adults by sex used in experiments exploring mating behavior when in A) Homogenous populations; B) 50% Heterogenous populations compared to the homogenous population (control); and C) 25% Heterogenous populations compared to the homogenous population (control) held in cages 128 x 84 x 84 cm mesh cages in a greenhouse maintained at 39 ± 4.1 °C and 44 ± 3.8% RH. Different letters indicate significance from Tukey HSD. LM = large males, SM = small males, LF = large females, SF = small females.

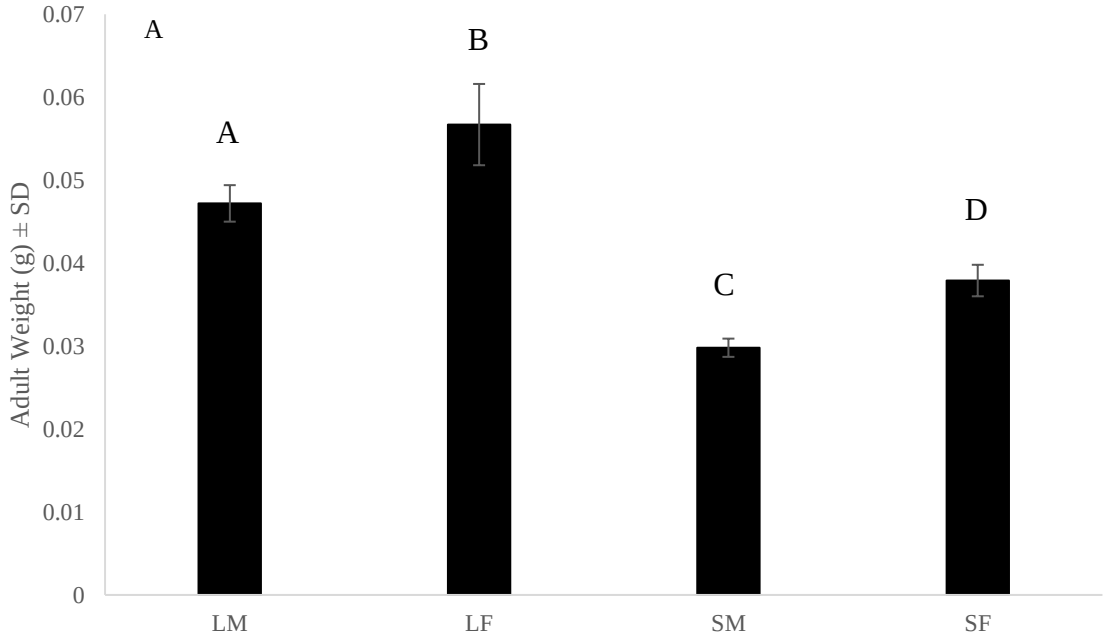
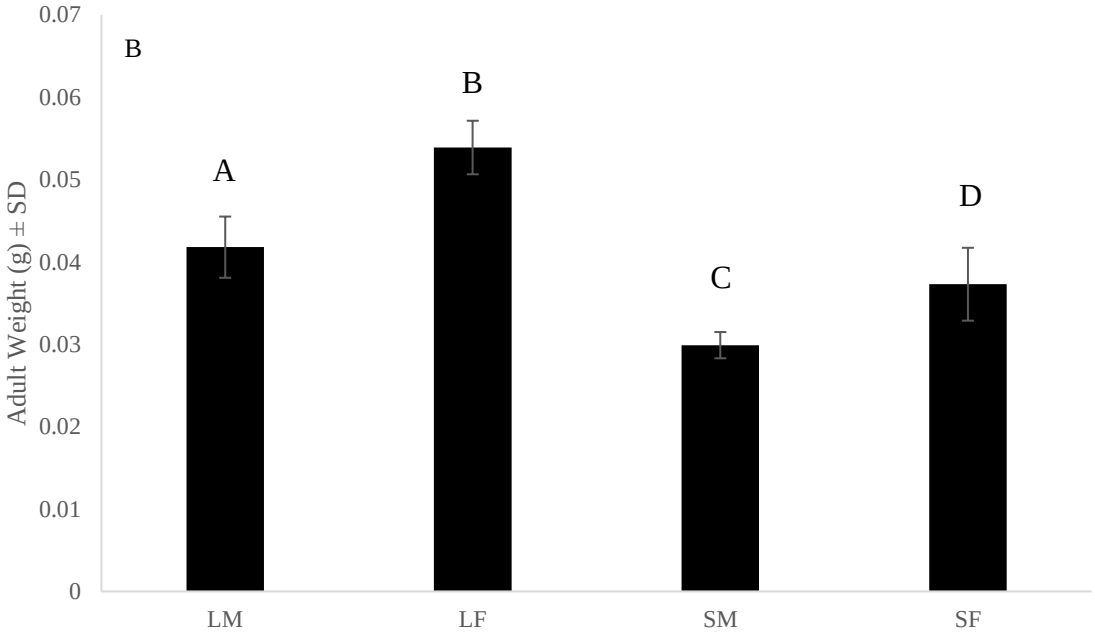
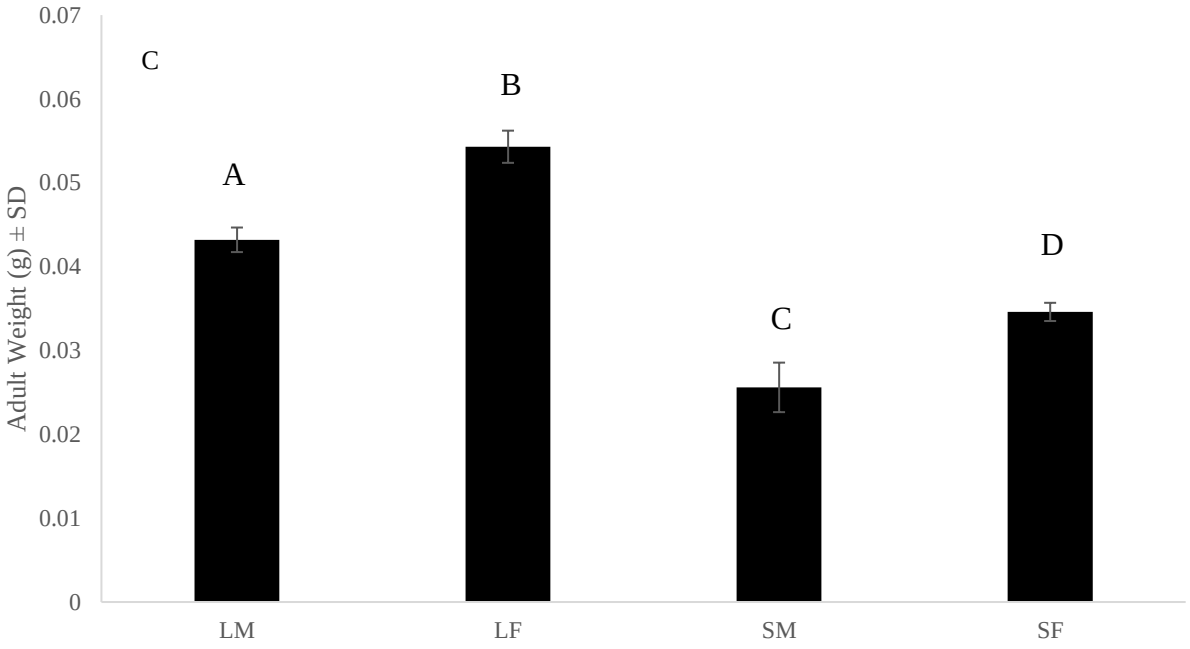


Figure 4.1 Continued





4.3.2 Homogenous populations (100%)

4.3.2.1 Mating observations

No significant ($t_1 = -0.260$, $P = 0.7971$) difference in number of successful matings between the large and small populations over the course of the experiment was determined (Table 4.1). Number of mating pairs by day was not significantly different ($F_{2, 2} = 0.0209$, $P = 0.9794$) between populations either (Supplementary Figure A.1). Number of failed matings did not significantly differ ($F_{1, 1} = 1.2161$, $P = 0.4689$) overall or by day ($F_{2, 2} = 4.2854$, $P = 0.1892$) between populations (Supplementary Figure A.2); neither did the overall number of multiple matings ($t_{23} = -0.9486$, $P = 0.3611$) or number of multiple matings by day ($F_{2, 2} = 1.5542$, $P = 0.2511$) (Supplementary Figure A.3).

Table 4.1: Mean life-history traits ± SD of large and small sized black soldier fly adults in homogenous populations (n = 4) held in cages 128 x 84 x 84 cm mesh cages in a greenhouse maintained at 39 ± 4.1 °C and 44 ± 3.8% RH.

	Mean ± SD	
	Large	Small
Successful Matings	68.00 ± 11.78 a[1]	62.00 ± 15.94 a
Failed Matings	10.00 ± 3.61 a	13.00 ± 2.97 a
Multiple Matings	10.00 ± 3.97 a	7.00 ± 3.86 a
# of Eggs	11500.00 ± 4010.70 a	10500.00 ± 5157.28 a
Hatch Rate (%)	43.00 ± 31.28 a	47.00 ± 20.16 a

[1] Lowercase letters indicate no significance between size classes ($p > 0.05$) from paired t-test.

4.3.2.2 Oviposition and hatch rate

Overall number of eggs (Table 4.1) or by day (Supplementary Figure A.4), produced by the large and small populations did not significantly differ ($H_7 = 1.6903$, $P = 0.1936$; $H_{11} = 4.7626$, $P =$

0.0924, respectively). Overall, or by day (Supplementary Figure A.5), hatch rate showed no significant difference ($H_7 = 0.2269$, $P = 0.6339$; $H_{11} = 1.2881$, $P = 0.5252$, respectively) between large and small populations (Table 4.1).

4.3.3 Heterogenous populations (50%)

4.3.3.1 Mating observations

A significance difference ($F_{3,3} = 104.373$, $P < 0.0001$) was determined for overall number of mating pairs across heterogenous and homogenous populations. Tukey HSD determined heterogenous populations with large males and small females had the greatest total mating pairs per observation (54.00 ± 4.21) in contrast to the small males and large females (36.00 ± 3.45) (Table 4.2). Number of mating pairs by day was significant ($F_{6,6} = 18.0476$, $P < 0.0001$) across heterogenous and homogenous populations (Supplementary Figure A.1). Tukey HSD determined large males paired with small females displayed the greatest total matings on days two (20.00 ± 1.94) and three (19.00 ± 2.87). Total failed matings observed differed overall ($F_{6, 17} = 9.98$, $P = 0.0020$) and by day (Supplementary Figure A.2). Number of multiple matings across heterogenous and homogenous populations differed overall ($H_{12} = 9.2389$, $P = 0.0263$). In heterogenous populations, large males with small females displayed the greatest number of multiple matings overall (14.00 ± 0.70), in contrast to small males with large females (5.00 ± 0.27) (Table 4.2). Number of multiple matings by day was significantly in heterogenous populations ($H_{19} = 14.1198$, $P = 0.0027$). Large males with small females had the greatest number of multiple matings (6.00 ± 1.73) on day two (Supplementary Figure A.3).

4.3.3.2 Oviposition and hatch rate

Number of eggs overall produced differed across heterogenous populations ($F_{3,3}$ = 8.373, P = 0.0019). The large females that mated with small males produced more eggs (15087.00 ± 3277.13) compared to small females mated with large males (5781.00 ± 2953.90) (Table 4.2). Number of eggs by day within heterogenous populations was significant (H_{11} = 16.332, P = 0.0002) (Supplementary Figure A.4). Overall hatch rate differed ($F_{3,3}$ = 4.687, P = 0.0181) across homogenous and heterogenous populations. Adults in homogenous populations had the greatest hatch rate overall (84.87 ± 3.65%) (Table 4.2). In addition, large females mated with small males had the lowest hatch rate overall (68.51 ± 2.66%). Hatch rate by day was not significant (H_7 = 1.2881, P = 0.5252) (Supplementary Figure A.5).

Table 4.2: Mean life-history traits ± SD of large and small black soldier fly adults for heterogenous (50%) populations (n = 6) compared to the homogenous population (control) held in 128 x 84 x 84 cm mesh cages in a greenhouse maintained at 41 ± 2.6 °C and 43 ± 5.8% RH. L = large adults, S = small adults, LM = large males, SM = small males, LF = large females, SF = small females.

	50% (mean ± SD)			
	L	S	LM[1] + SF	SM[2]+ LF
Successful Matings	25.00 ± 5.08 C[3]	24.00 ± 3.88 C	54.00 ± 4.21 A	36.00 ± 3.45 B
Failed Matings	9.00 ± 2.01 A	6.00 ± 1.43 B	9.00 ± 1.21 A	8.00 ± 1.67 A
Multiple Matings	5.00 ± 0.37 B	2.00 ± 0.64 B	14.00 ± 0.70 A	5.00 ± 0.27 B
# of Eggs	18150.00 ± 4654.79 A	8474.00 ± 4461.54 C	5781.00 ± 2953.90 B	15087.00 ± 3277.13 B
Hatch Rate (%)	77.67 ± 4.43 B	84.87 ± 10.71 A	76.57 ± 4.34 A	68.51 ± 2.66 B

[1] LM+SF = large males with small females
[2] SM+LF = small males with large females
[3] Capital letters indicate significance between treatments from Tukey HSD analysis

4.3.4 Heterogenous populations (25% each sex and density)

4.3.4.1 Morphometrics

MANOVA of adult morphometrics measured was significant across populations (Pillai = 0.86, $F_{7, 113}$ = 124.89, $P < 0.0001$) (Table 4.3A). Following up with an ANOVA, wing length, antennal length, leg length, head width, abdominal length, and eye length were significantly different ($P < 0.0001$) across populations. However, external genitalia lengths did not differ ($F_{7, 118} = 0.0007$, $P = 0.9800$) between adult sizes, but differed between sexes ($P < 0.0001$) (Table 4.3B).

Table 4.3A: ANOVA table of selected black soldier fly adult morphometrics. A MANOVA was implemented before ANOVA analysis. Black soldier flies were reared in a walk-in growth chamber set at 29.5 ± 0.7°C with 66 ± 5.6% RH and 14:10 L:D photoregime.

ANOVA Statistics					
Morphometrics[1]	Variables	DF	MS	F	P-value
WL	Size	1	93.51	189.03	< 0.0001
	Sex	1	23.15	21.22	< 0.0001
ABL	Size	1	83.35	133.41	< 0.0001
	Sex	1	8.47	6.72	0.0106
LL	Size	1	118.16	242.74	< 0.0001
	Sex	1	13.80	10.06	0.0019
HW	Size	1	10.26	210.75	< 0.0001
	Sex	1	1.01	7.97	0.0055
EL	Size	1	7.54	553.57	< 0.0001
	Sex	1	0.35	4.78	0.0306
AL	Size	1	12.53	178.73	< 0.0001
	Sex	1	2.38	15.25	0.0001
EG	Size	1	0.00	0.00	0.98
	Sex	1	1.13	1885.9	< 0.0001

[1] WL = wing length, ABL = abdominal length, LL = leg length, HW = head width, EL = eye length, AL = antennal length, EG = external genitalia length.

Table 4.3B: Morphometrics (mm) ± SD (n = 120) of black soldier fly adults in a walk-in growth chamber set at 29.5 ± 0.7°C with 66 ± 5.6% RH and 14:10 L:D photoregime.

Sex (Body Size) and Mean Length or Width (mm) ± SD				
Morphometrics[1]	LM[2]	SM	LF	SF
WL	9.57 ± 0.06 B[3]b[4]	7.45 ± 0.16 Dd	10.12 ± 0.08 Aa	8.68 ± 0.06 Cc
ABL	8.02 ± 0.10 Bb	6.45 ± 0.11 Dd	8.71 ± 0.12 Aa	6.83 ± 0.18 Cc
LL	8.87 ± 0.06 Bb	6.67 ± 0.10 Dd	9.31 ± 0.10 Aa	7.68 ± 0.26 Cc
HW	3.48 ± 0.07 Bb	2.95 ± 0.07 Dd	3.75 ± 0.05 Aa	3.12 ± 0.04 Cc
EL	1.76 ± 0.04 Bb	1.30 ± 0.02 Dd	1.91 ± 0.05 Aa	1.37 ± 0.03 Cc
AL	3.58 ± 0.04 Bb	3.05 ± 0.05 Dd	3.95 ± 0.05 Aa	3.22 ± 0.04 Cc
EG	0.56 ± 0.02 Bb	0.57 ± 0.02 Bb	1.18 ± 0.03 Aa	1.18 ± 0.02 Aa

[1] WL = wing length, ABL = abdominal length, LL = leg length, HW = head width, EL = eye length, AL = antennal length, EG = external genitalia length.
[2] LM = large male, SM = small male, LF = large female, SF = small female
[3]Capital letters indicate significance between sizes from ANOVA.
[4] Lowercase letters indicate significance between sexes.

4.3.4.2 Mating observations

Overall number of mating pairs within the heterogenous populations was significantly different ($F_{5,5}$ = 6.4950, P = 0.0038). Large males with small females had the greatest overall number of mating pairs (24.00 ± 7.13) compared to the number of small males with large female mating pairs (9.00 ± 2.96) (Supplementary Figure A.1). A significant difference (H_{17} = 45.5255, P = 0.0002) between day and observed mating pairs in the heterogenous populations, where mating frequency of small males with large females was lowest compared to the homogenous small males on days one and two, was determined. Overall number of failed mating attempts did not differ ($F_{5,5}$ = 0.6308, P = 0.6802) between heterogenous and homogenous populations. However, when separating failed matings by day, small males attempting to mate with females increased on day two (H_{17} = 30.0070, P = 0.0263) (Supplementary Figure A.2). Overall number of multiple matings differed (H_{16} = 24.9996, P = 0.0198) in heterogenous populations with large males displaying the lowest number of multiple matings with small females (1.00 ± 0.47) (Figure 4.2). When separated

by day, multiple matings between small males and small females were not observed on day two unlike large males and large females (Supplementary Figure A.3). Male-to-male aggression showed overall significance ($F_{4,10}$ = 3.9876, P = 0.0346) in heterogenous populations. When observing male-to-male aggression by day, there was a significance (H_{19} = 48.5401, P = 0.0002) where homogenous populations of large males exhibited the highest number of male-to-male aggression (9.00 ± 1.67) on day one only (Figure 4.3).

Figure 4.2: Comparisons of 25% heterogenous population compared to the homogenous population (control) experiment of black soldier fly adults mating observations ± SD in 128 x 84 x 84 cm cages in a greenhouse maintained at 40 ± 1.1 °C and 50 ± 3.2% RH. A) Overview of mating observations, B) Successful matings only. L = large adults, S = small adults, LM = large males, SM = small males, LF = large females, SF = small females.

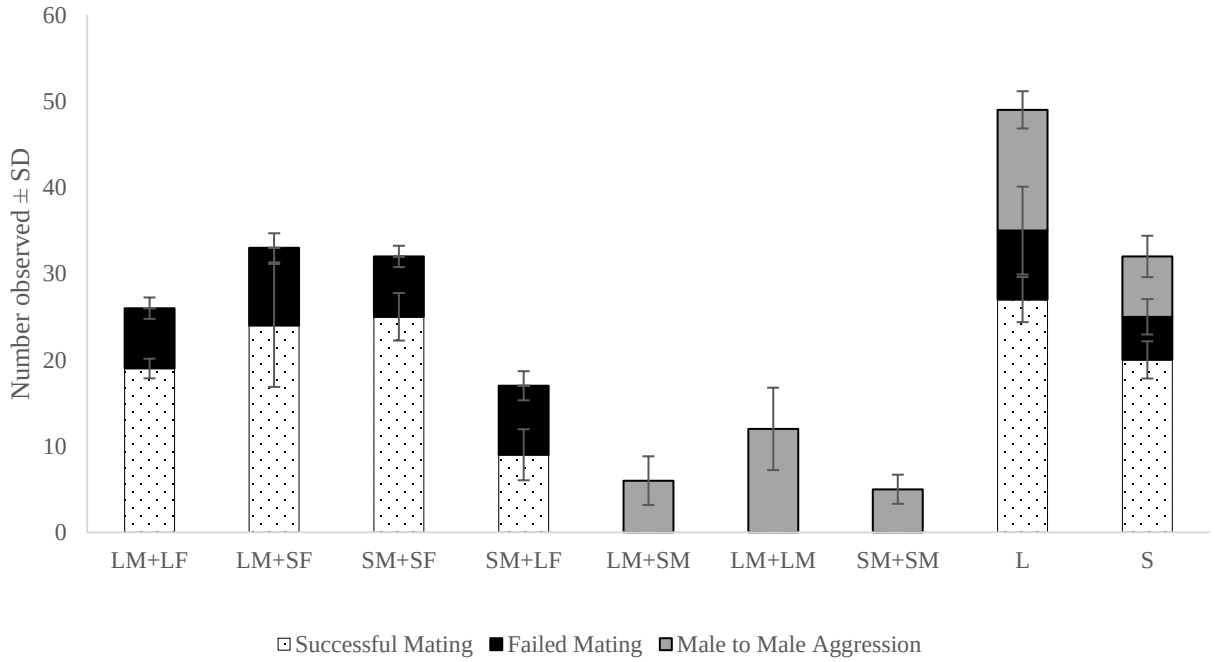
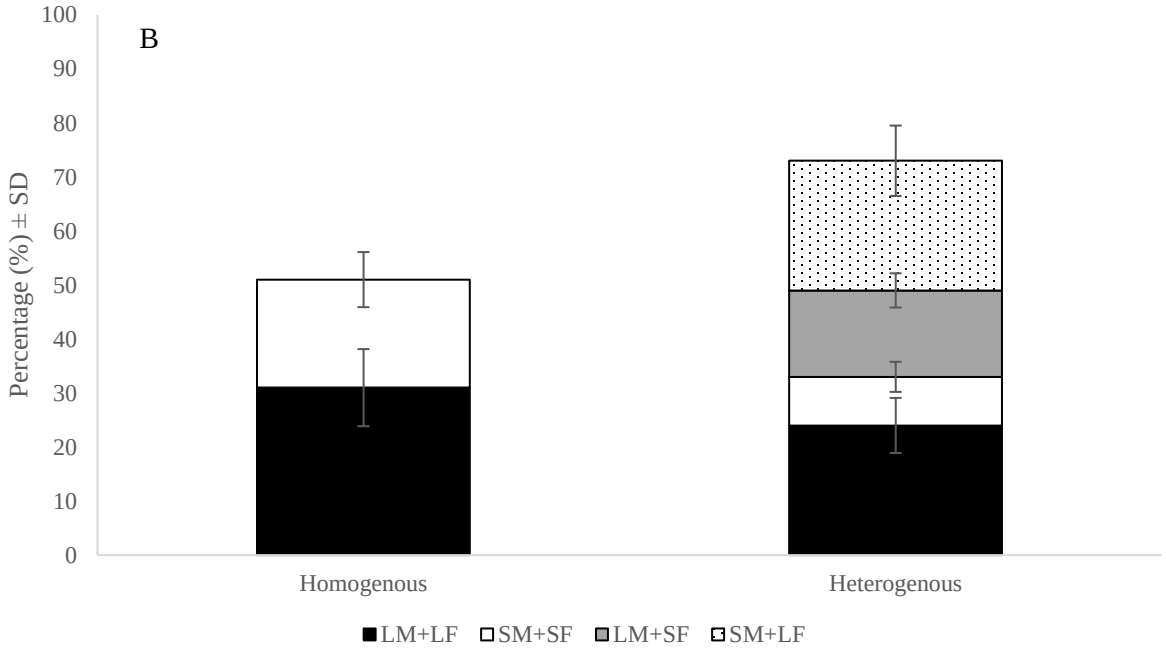


Figure 4.2 Continued

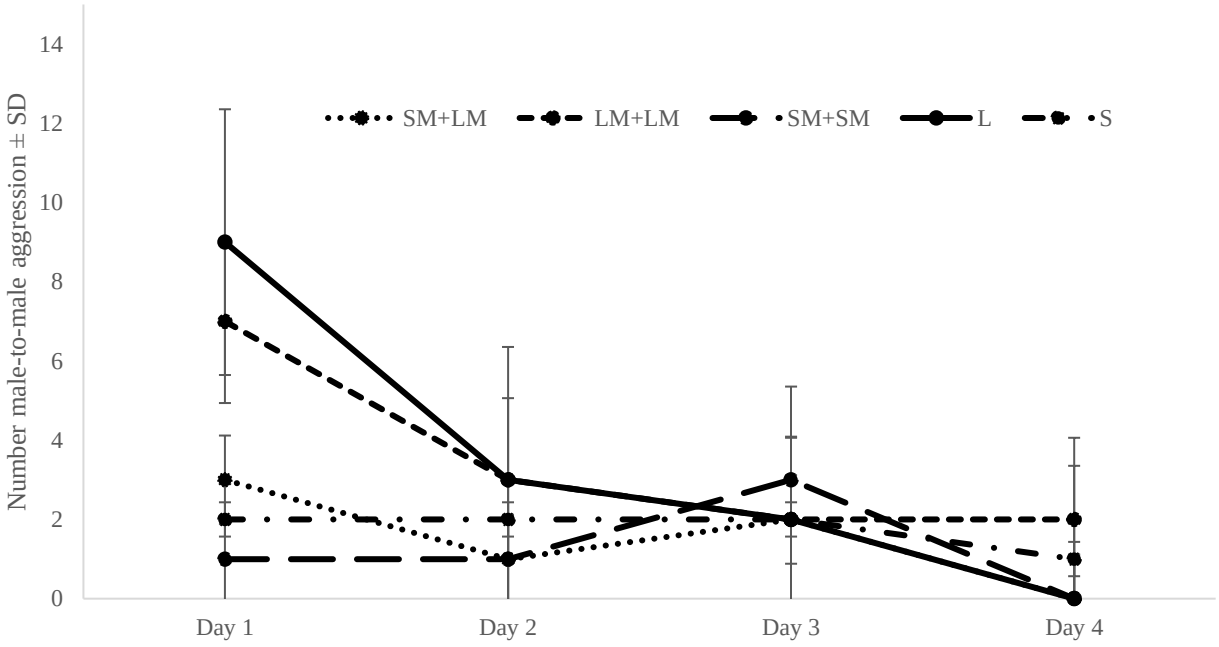


4.3.4.3 Oviposition and hatch rate

Total number of eggs significantly differed ($H_9 = 8.4797$, $P = 0.0144$) between heterogenous and homogenous populations where heterogenous populations produced more eggs (31384.00 ± 1898.65) than the homogenous populations of small adults (7082.00 ± 4005.61). Number of eggs by day showed significance ($H_{13} = 19.5661$, $P = 0.0121$) (Supplementary Figure A.4). Heterogenous and homogenous populations of large adults produced the most eggs on day one (17744.00 ± 3050.05 and 13610.0 ± 1726.52, respectively) compared to the homogenous populations of small adults. Overall hatch rate showed no significance ($H_9 = 3.0723$, $P = 0.2152$) across heterogenous and homogenous populations. Hatch rate by day also was not significantly different ($H_{13} = 13.8201$, $P = 0.0866$) (Supplementary Figure A.5). Heteroscedasticity was significant ($F_{2, 15} = 7.5511$, $P = 0.0053$) across treatments and considered with the associated

analyses applied. In fact, the variability in egg hatch rate was forty times greater than the large homogenous populations, and 40% greater than the small homogenous populations (Supplementary Table A.1). A likelihood ratio analysis determined no difference between large males securing a mate with large or small females. However, small males were likely to mate with small females ($P < 0.05$) compared to large females (Supplementary Figure A.6).

Figure 4.3: Total number of male-to-male aggression events ± SD observed per day in populations of 25% heterogenous population experiment of black soldier fly adults mating observations in 128 x 84 x 84 cm cages in a greenhouse maintained at 40 ± 1.1 °C and 50 ± 3.2% RH. L = large adults, S = small adults, LM = large males, SM = small males, LF = large females, SF = small females.



4.4 Discussion

Homogenous populations (100%) of large and small black soldier fly adults showed no differences in total number of mating pairs or egg production. However, in the subsequent experiments,

homogenous populations of large adults produced 72 and 100% more eggs than the small adults in the homogenous populations. For 50% heterogenous populations, large males paired with small females displayed higher mating success and produced more eggs than the inverse pairing; however, large females mated with small males produced more eggs than the inverse pairing but had an 8% reduction in hatch rate compared to the inverse mate pair. Heterogenous populations (25%) showed higher frequencies of large males securing small female mates compared to small males securing large female mates (Supplementary Table A.1). Although overall number of failed matings did not differ, male-to-male aggression was frequent with larger males (Figure 4.3).

4.4.1 Effects of body size on mating behavior

Body size impacted mating success of the black soldier fly. Similar results have been found in other species such as *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae) (Ekanayake, Clarke, & Schutze, 2017) and *Cotesia urabae* (Hymenoptera: Braconidae) (Avila et al., 2017), where large males had higher numbers of successful matings compared to small males. Observations of swarming *Anopheles freeborni* (Aitken) (Diptera: Culicidae) males in a natural environment showed that females favored larger males (Yuval, Wekesa, & Washino, 1993). Another field observation study of *Drosophila buzzatii* (Patterson and Wheeler) found that sample sizes of mated males consisted of mostly large males, suggesting that large flies are most likely to secure mates compared to smaller males (Santos et al. 1988).

Large black soldier fly males possibly overcame female avoidance (i.e., sexual conflict). As discussed previously, wing fanning by male black soldier flies was reported as a significant part of courtship behavior to obtain females (Giunti et al., 2018). There may be a possible link between wing fanning behavior and mating success of adult males based on size (i.e., sexual

selection). Furthermore, when separating mating observations by day within the 50% heterogenous population, small males paired with large females displayed higher counts of failed matings on day one compared to large males with small females on the same day (Supplementary Figure A.2). This hypothesis is plausible as wing vibration courtship behavior has also been reported in *Bactrocera oleae* (Rossi) (Diptera: Tephritidae), where large males that had successfully mated displayed faster wing vibrations than smaller males (Benelli et al., 2016). In the 25% heterogenous treatment, small black soldier fly males were more likely to mate with females of equal sizes, considering the number of successful and failed matings were almost similar in marked pairs of small males and large females. Such results suggest that unlike the smaller males, large males are more persistent, which leads to greater likelihood of securing a mate.

Indeed, adult size is clearly an important factor regulating mating success. However, we would like to emphasize for this experiment that while we designated our populations as large or small, it should be noted that males within a population were consistently smaller than the females. We hypothesize the ratio of body size between males and females is a major factor regulating mating success. If the ratio falls below a hypothetical threshold, males are less likely to secure a mate. For example, number of successful matings in the copepod, *Diaptomus birgei* (Marsh) (Calanoida: Diaptomidae) were greatest (>60%) when the sex size ratios ranging from 1.15 to 1.23 (female: male lengths) (Grad and Maly, 1988). Below, or above, those thresholds resulted in a 20% decrease in number of mating successes.

Larger black soldier fly males displayed a greater frequency of aggression towards other males, particularly during the first day of mating observations (Figure 4.3). This behavior has been found in other Diptera species. For example, males of three species of crane flies (Diptera: Tipulidae) display acts of aggression as a form of mate guarding (Adler & Adler, 1991). In

addition, male-male aggression could be a major contributing factor that selects for dominant males in lekking behavior systems. Similar behaviors have been found in other species such as *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) (Arita & Kaneshiro, 1989; Whittier, Nam, Shelly, & Kaneshiro, 1994). However, male-male aggression can possibly affect longevity for other fly species, which can be costly (Hooper et al., 2017).

4.4.2 Effects of body size on oviposition and hatch rate

Adult female size was an indicator of egg production. This correlation is well known in other insect species (Calvo & Molina, 2005; Morimoto, Nguyen, Tabrizi, Ponton, & Taylor, 2018; Thornhill & Alcock, 1983). In the current study, larger black soldier fly females produced approximately 50% more eggs (Table 4.2). Similar results were found in large females of *Aedes aegypti* (L.) (Diptera: Culicidae) that produced 50% more eggs than small females, but number of viable eggs varied between small and large females mated with small and large males (Dieng et al., 2016).

Egg hatch was highly variable for the heterogenous populations of black soldier fly. Reasons for variable egg viability in heterogenous black soldier fly populations could be explained by sperm load (Neems, J. Lazarus, & McLachlan, 1998) and sperm viability (Blay & Yuval, 1999) in different sized males. In polyandrous species such as *Dryomyza anilis* (Fallén) (Dryomyzidae), fertilization rate of males decreases by 8-10% when mated with a non-virgin female (Otronen, 1994). The possibility of high variability in egg hatch in this study may have resulted from multiple mating attempts by large males. Although multiple mating frequencies for the heterogenous population experiments accounted for 10-14% of mating successes, large males displayed successful remating actions more than small males (Supplementary Figure A.3). Other

explanations for variability in egg production could include factors such as age of adults (Moreau, Monceau, & Thiery, 2016) and malnourishment (Bonduriansky & Head, 2007).

4.4.3 Conclusions and industry recommendations

For this study, the question exploring how male body size influences mating success of the black soldier fly was explored. Large males displayed higher counts of mating success and male-male aggression in populations comprised of variably sized adults. Large females were more fecund than small females, but their hatch rates were influenced by the size of the adult male with whom they mated.

Several limitations of this study should be taken into consideration for future studies. First, initiation of larval population densities was staggered on a weekly basis to promote similar adult emergence times. Thus, males emerge two days before females, and in order to reach equal sex ratio, black soldier fly adults were of mixed ages during observation studies. Second, in an artificial rearing system (i.e., indoor) for black soldier flies, egg production increased with increasing adult density (Hoc et al., 2019). Therefore, future studies should explore variable adult densities and sex ratios to optimize egg production.

From an industrial standpoint, mass production facilities should evaluate their practices to ensure production of males and females, respectively, of consistent size. Furthermore, while raising larvae at low densities means fewer adults produced, they can develop 50% faster than at higher densities and require less feed (Jones & Tomberlin, 2019), which means overall increase in production with less investment in space and larval food, respectively. Most importantly, while populations comprised of mixed adult size yielded significantly more eggs (38% more than homogenous; Table 4), the hatch variability (i.e., 40X greater variability than in the homogenous

populations; Supplementary Table A.1) translates into an inability to predict larval output. Therefore, we recommend standardized production of adults in colony to minimize hatch variability. Once established, industry can explore options for optimizing total number of eggs produced.

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CHAPTER V

CONCLUSIONS

5.1 Summary of results

Overall, the research I conducted for my dissertation aimed to investigate the link between larval competition of the black soldier fly, *Hermetia illucens* (L.), (Diptera: Stratiomyidae) and the mating success of resulting adults based on size. In chapter two, larval competition of four different densities (500, 1000, 1500, and 2000 larvae/4L with inversely rated feed rates of (0.027, 0.036, 0.054, and 0.108 g/larva/day) resulted in the lowest larval density (500) producing heavier individuals than the greatest larval density (2000) for larvae (15%), prepupae (19%), and adult (35%) stages. In addition to weights, larvae reared at the lowest density developed 63% faster than those reared at the highest density. For chapter three, a study evaluating an acrylic paint-marking technique with four colors (green, gold, red, and white) on the black soldier fly in a greenhouse setting was conducted. I determined this marking technique had no significant impact on adult longevity or mating success. In chapter four, I used findings from chapters two and three to determine the impact of black soldier fly adult sizes on mating success. The key findings were: (1) multiple matings accounted for 10-14% of successful matings in heterogenous populations; (2) in homogenous populations, there were no significant differences in mating frequency, egg production, or egg viability; (3) in 50% heterogenous populations where sexes and densities were crossed (large males with small females and vice versa), large males exhibited 100% higher mating frequencies than in the homogenous populations. Large females produced 3x more eggs than small females, but with an 8% lower hatch rate; and (4) heterogenous populations with 25% of each density and sex within each treatment resulted in 50% more successful matings than in homogenous populations (controls). Large males displayed 2x more aggression towards smaller

males, but very little change was found towards similar sized males. In addition, approximately 30 to 400% more eggs were produced. However, the variability in egg hatch was forty times greater than the homogenous large population and 40% greater than the small homogenous population. The likelihood of small males mating with small females was greater than small males mating with large females. In summary, larval competition of the black soldier fly—in terms of different larval densities feeding on a standard diet—affects their larval development time, size, and adult mating success. Furthermore, while mixing adult sizes resulted in greater number of eggs deposited, the associated variability in numbers deposited and corresponding hatch rate severely impact predictions of mass production (i.e., some days a large number of eggs are collected and hatch, while other days opposing results are determined). I recommend adult size (*i.e., specifically, male to female ratio be consistent as males produced in a cohort are typically smaller than females*) be standardized so as to minimize variability across generations. Introducing variability in the male to female size ratio translates into unpredictable production of fertile eggs.

5.2 Rationale and application

The concern for global food security remains an important topic due to the human population predicted to exceed 9 billion by the year 2050 (FAO 2013). As a result, high demand for livestock and aquaculture production will greatly increase manure waste from confined animal facilities that produce noxious emissions (FAO 2011). Globally, humans produce about 1.3 billion tons of food waste per year, also resulting in increased noxious landfill emissions such as ammonia, which potentially threatens human health (FAO 2011). One possible solution is to utilize insects, particularly the black soldier fly, to help combat these issues (van Huis 2013).

As generalist feeders, interest in mass production of the black soldier fly continues to rise due to larvae feeding on multiple decomposing organic wastes such as animal manure (Sheppard et al. 1998, Beskin et al. 2018, Miranda et al. 2019), human feces (Banks et al. 2014), food waste (Nguyen et al. 2015, Parra Paz et al. 2015), and even animal tissue (Tomberlin et al. 2004, Pujol-Luz et al. 2008). In addition, the high protein content (~40%) of black soldier fly larvae and prepupae is an important factor as alternative feed for livestock and aquaculture (Wang and Shelomi 2017). However, fully exploring various feed rates and larval densities remains to be a critical component of optimizing mass production of the black soldier fly. For example, most studies on larval density and the associated the life history traits were conducted on a laboratory scale (Myers et al. 2008, Diener et al. 2009, Banks et al. 2014, Parra Paz et al. 2015, Barragan-Fonseca et al. 2018). Although this study was also completed on a laboratory scale, higher larval densities of 500, 1000, 1500, and 2000 larvae/4L displayed similar findings of the previously mentioned, regarding feed rates of 0.108 g/larva reducing development time and producing larger black soldier flies across all life stages. Therefore, the industry should consider testing scaled densities with similar feed rates of other available resources (i.e., food waste and manure) to determine the best larval rearing methods of the black soldier fly in order to reduce maintenance costs.

The knowledge of black soldier fly mating behavior has been limited. Adult black soldier flies are known to mate under natural sunlight (Tomberlin and Sheppard 2002). Furthermore, optimizing egg production for colony maintenance in mass production facilities is not well known (Hoc et al. 2019). Previous studies suggested that large body sizes were important for mating success of *Hermetia* species (Tingle et al. 1975, Alcock 1990). A recent study detailed courting behavior of the male black soldier fly, where they display wing-fanning behavior for female mate

selection (Giunti et al. 2018). To combine previous findings with this study in a greenhouse setting, large males displayed higher counts of male-to-male aggression against other males of similar and smaller sizes and had higher mating success with females. Although rare, large males have a higher chance of mating again when compared to smaller males. These findings suggest that large body sizes in adult males increases chances of mating success. Overall, large females produced more viable offspring than small females. In summary, mass production facilities should consider that low larval densities of black soldier flies develop faster and produce large adults and more eggs. Depending on what the industry focuses on—in terms of black soldier fly colony maintenance, or larval production—mass rearing facilities should test what is optimal and less costly.

5.3 Potential limitations and future research implications

Translating laboratory studies to industry standards is challenging (Piccinno et al. 2016). Therefore, several limitations of this study should be taken into consideration. First, the entire study utilized a standard Gainesville house fly, *Musca domestica*, L. (Diptera: Muscidae) diet (Hogsette 1992) for a black soldier fly laboratory colony that produced most generations on the grain-based diet since 1998. Higher-scale larval density studies should explore different resources such as manure (Diener et al. 2009) and food waste (Parra Paz et al. 2015) in order to accurately provide new detailed data on colony maintenance in other mass rearing facilities that do not have access to the Gainesville house fly diet. Furthermore, future research should investigate the impact of larval nutrition (i.e. macronutrients), rather than density, on black soldier fly mating success. A few studies have investigated larval diet on adult longevity (Tomberlin et al. 2009; Cammack and Tomberlin 2017); however, future studies should also consider varying macronutrient ratios of larval diets affecting morphometrics of both sexes (Bonduriansky 2007; Hooper et al. 2017). Such

findings should be explored with the black soldier fly, where the impact of nutritional quality of larval diets affects adult mating success.

Mating studies for this project were completed in a greenhouse setting, and susceptible to temporary weather conditions (i.e., thunderstorms). Few other studies explored black soldier fly mating behavior under natural light (Tomberlin and Sheppard 2001, Tomberlin and Sheppard 2002, Park et al. 2010, Zhang et al. 2010), but many black soldier fly mass production facilities rely on indoor mating for controlled abiotic settings and to reduce the impact of unfavorable climates (Zhang et al. 2010, Oonincx et al. 2016, Hoc et al. 2019). Additionally, these studies were conducted with two-hundred adult black soldier flies per cage. Although Hoc et al. (2019) did not conduct mating behavior observations, the authors suggested that higher adult densities with female-biased sex-ratios produced more eggs. In high density adult populations for other species, male-biased sex ratios will result in higher counts of a reproductive strategy known as mate guarding of females (Baxter et al. 2015). However, increased mate guarding behavior and male-to-male aggression could result in losing a potential mate, or multiple mating opportunities, as seen in *Dactylolabis montana* (Osten Sacken), *Limonia simulans* (Walker), and *Antocha saxicola* (Osten Sacken) (Adler and Adler 1991). Therefore, evaluating different sex ratios on black soldier fly lekking behavior would be beneficial to link the biological understandings of this species and providing more detailed data on mating interactions and favorable egg production for industrial standards.

The acquisition of black soldier flies for mating studies required eggs to be collected at different times for both larval densities to have similar adult emergence times, resulting in different generations responding to experimental manipulations. Therefore, future studies should consider investigating the response of the offspring from the parental generations. For example,

(Bonduriansky and Head 2007) manipulated the macronutrient ratio of the artificial diets for the stalk fly *Telostylinus angusticollis* (Enderlein) (Diptera: Neriidae) parental and offspring generations and found that the offspring of different parental diet qualities developed at similar rates when crossed with varying larval diets.

A major limitation of the black soldier fly mating studies, including those in my dissertation, is that mixed ages of adults were used. Although my experiments used virgin flies, there is a possibility that age plays a key role in mating success. For example, older medfly males, *Ceratitis capitate* (Wiedmann) (Diptera: Tephritidae) that were given low protein diets during their immature and adult stages did not successfully mate with younger females, suggesting that age and nutrition play a key role in mating success (Roriz and Joachim-Bravo 2013). Hopefully, my research will result in novel applications as well as the development of research built on my results exploring in greater detail intraspecific competition of the black soldier fly and its effects on mating success for mass production facilities. The black soldier fly can also be used as a model organism for questioning the trade-off between intraspecific competition and adult mating success.

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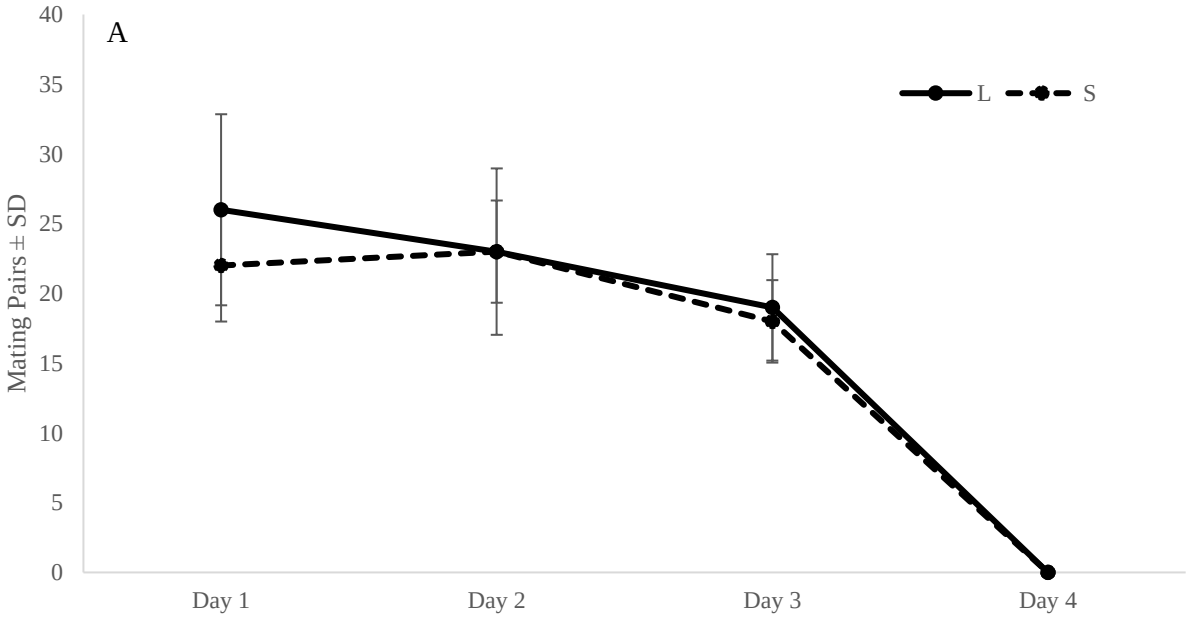
APPENDIX

Supplementary Figure A.1: Mean number of black soldier fly mating pairs ± SD observed per day for A) homogenous populations (n = 4); B) 50% heterogenous populations compared to the homogenous population (control) (n = 6); and C) 25% heterogenous populations compared to the homogenous population (control) (n = 6) held in 128 x 84 x 84 cm mesh cages in a greenhouse maintained at 39 ± 4.1 °C and 44 ± 3.8% RH. L = large adults, S = small adults, LM = large males, SM = small males, LF = large females, SF = small females.



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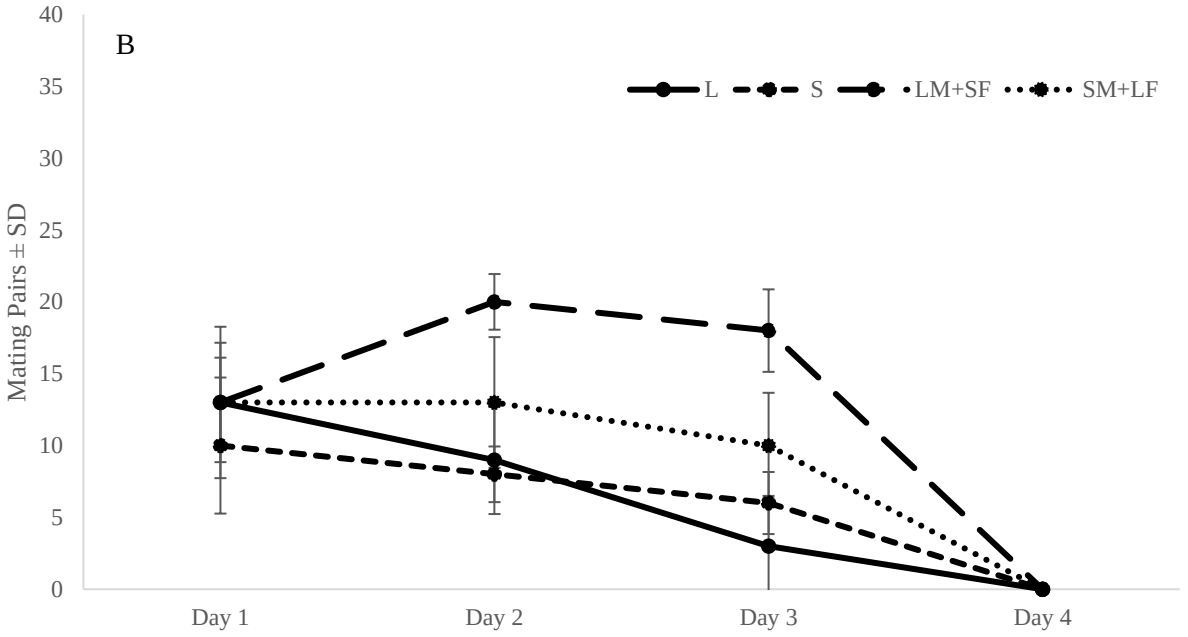
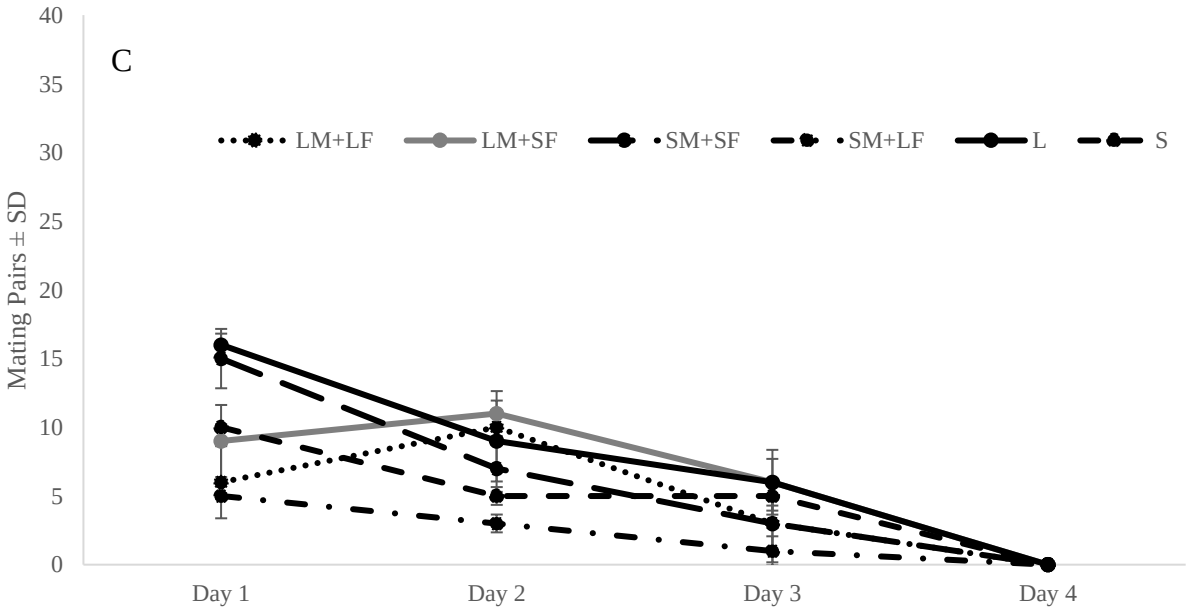
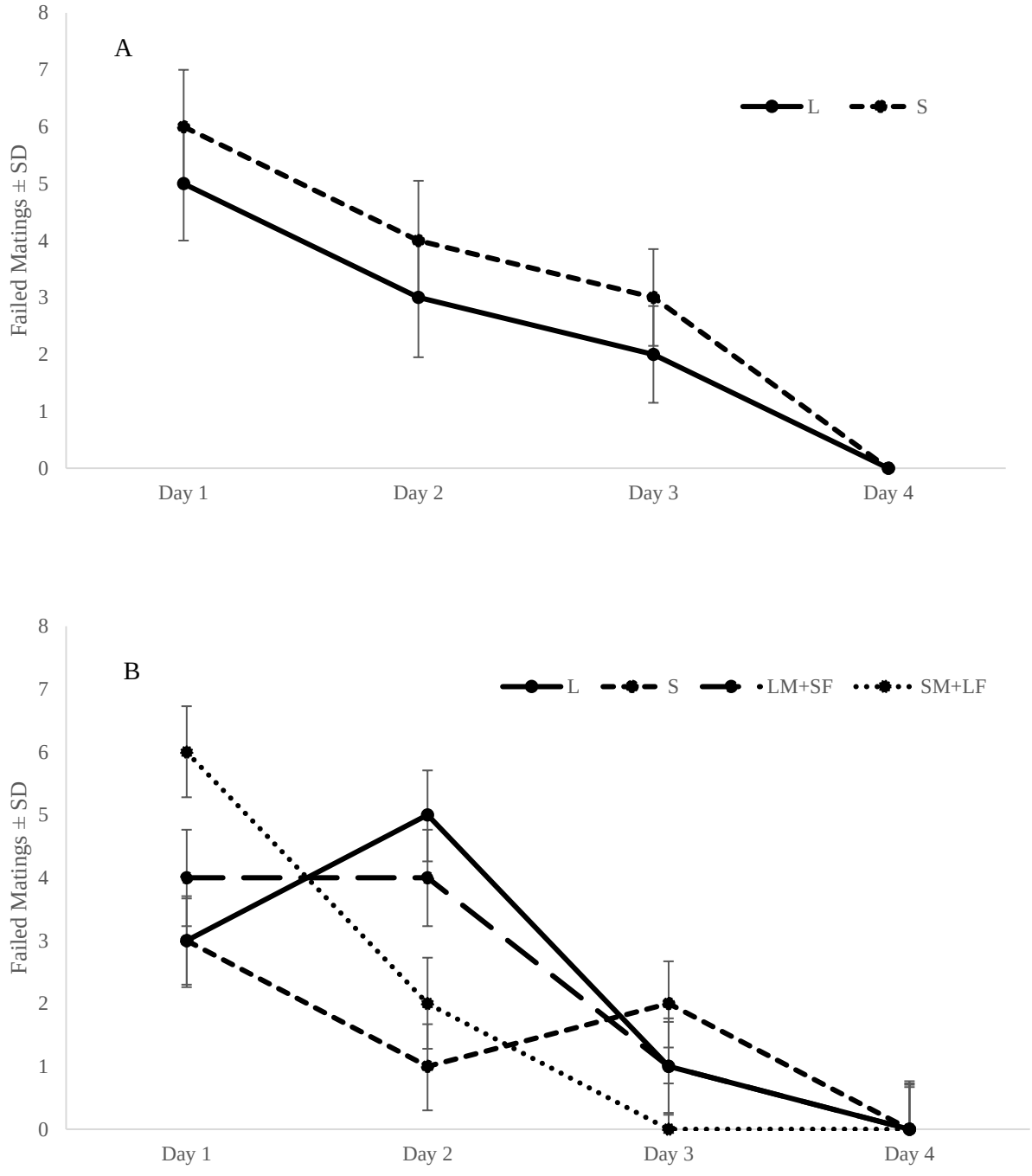




Supplementary Figure A.2: Mean number of black soldier fly failed matings ± SD (n = 4) observed per day for homogenous A) homogenous populations (n = 4); B) 50% heterogenous populations compared to the homogenous population (control) (n = 6); and C) 25% heterogenous populations compared to the homogenous population (control) (n = 6) held in 128 x 84 x 84 cm mesh cages in a greenhouse maintained at 39 ± 4.1 °C and 44 ± 3.8% RH. L = large adults, S = small adults, LM = large males, SM = small males, LF = large females, SF = small females.



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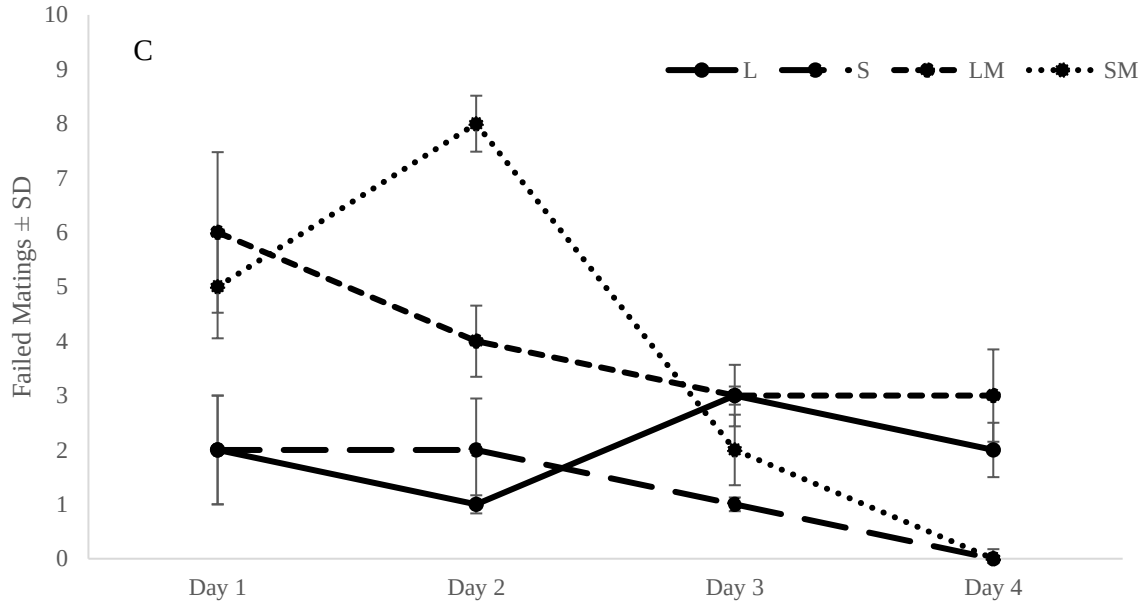
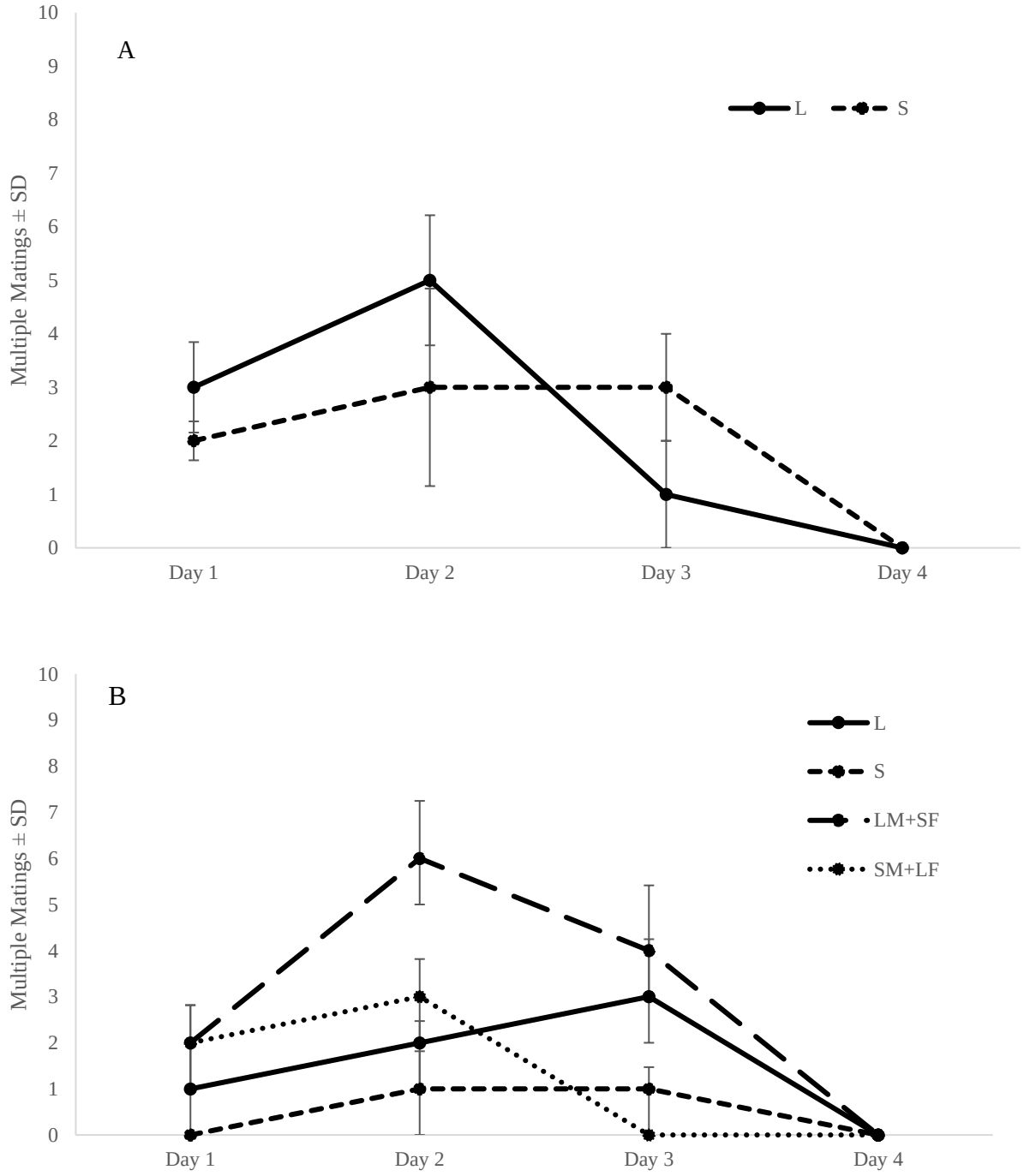


Supplementary Figure A.3: Mean number of multiple matings ± SD (n = 4) observed per day for A) homogenous populations (n = 4); B) 50% heterogenous populations compared to the homogenous population (control) (n = 6); and C) 25% heterogenous populations compared to the homogenous population (control) (n = 6) held in 128 x 84 x 84 cm mesh cages in a greenhouse maintained at 39 ± 4.1 °C and 44 ± 3.8% RH. L = large adults, S = small adults, LM = large males, SM = small males, LF = large females, SF = small females.



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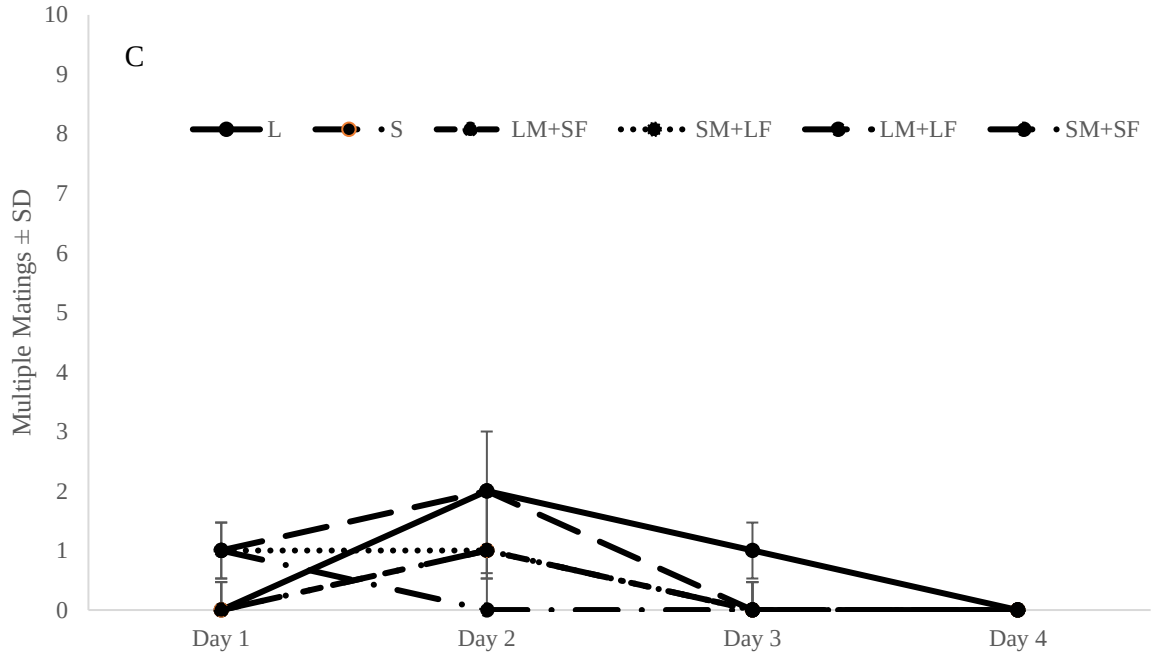
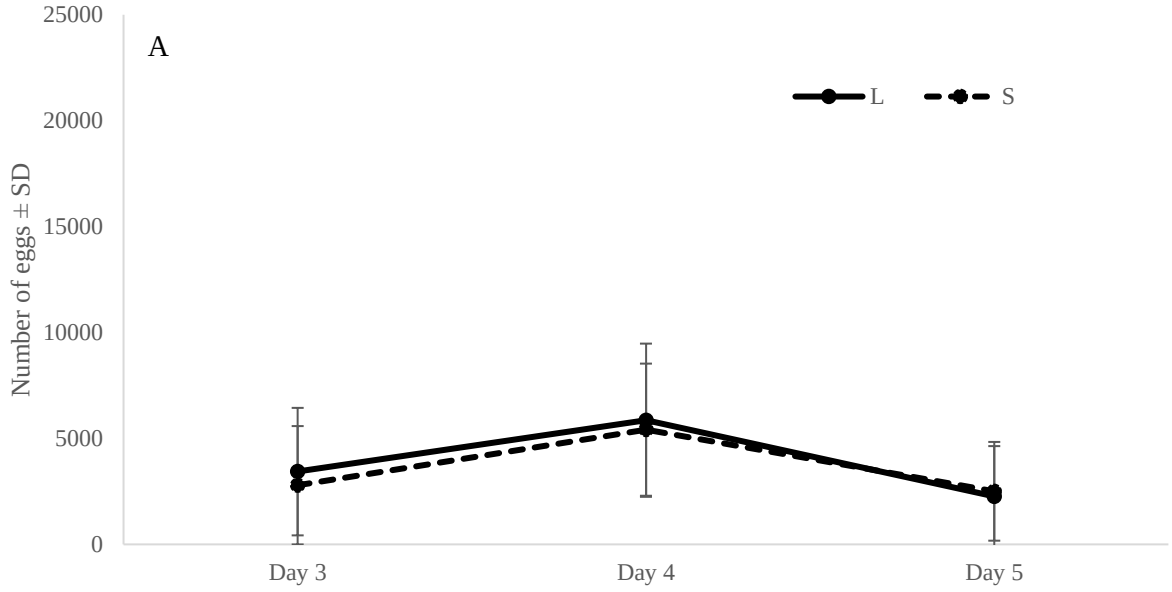


Supplementary Figure A.4: Mean number of eggs ± SD (n = 4) collected consecutively after second day of mating observation A) homogenous populations (n = 4); B) 50% heterogenous populations compared to the homogenous population (control) (n = 6); and C) 25% heterogenous populations compared to the homogenous population (control) (n = 6) held in 128 x 84 x 84 cm mesh cages in a greenhouse maintained at 39 ± 4.1 °C and 44 ± 3.8% RH. L = large adults, S = small adults, LM = large males, SM = small males, LF = large females, SF = small females.



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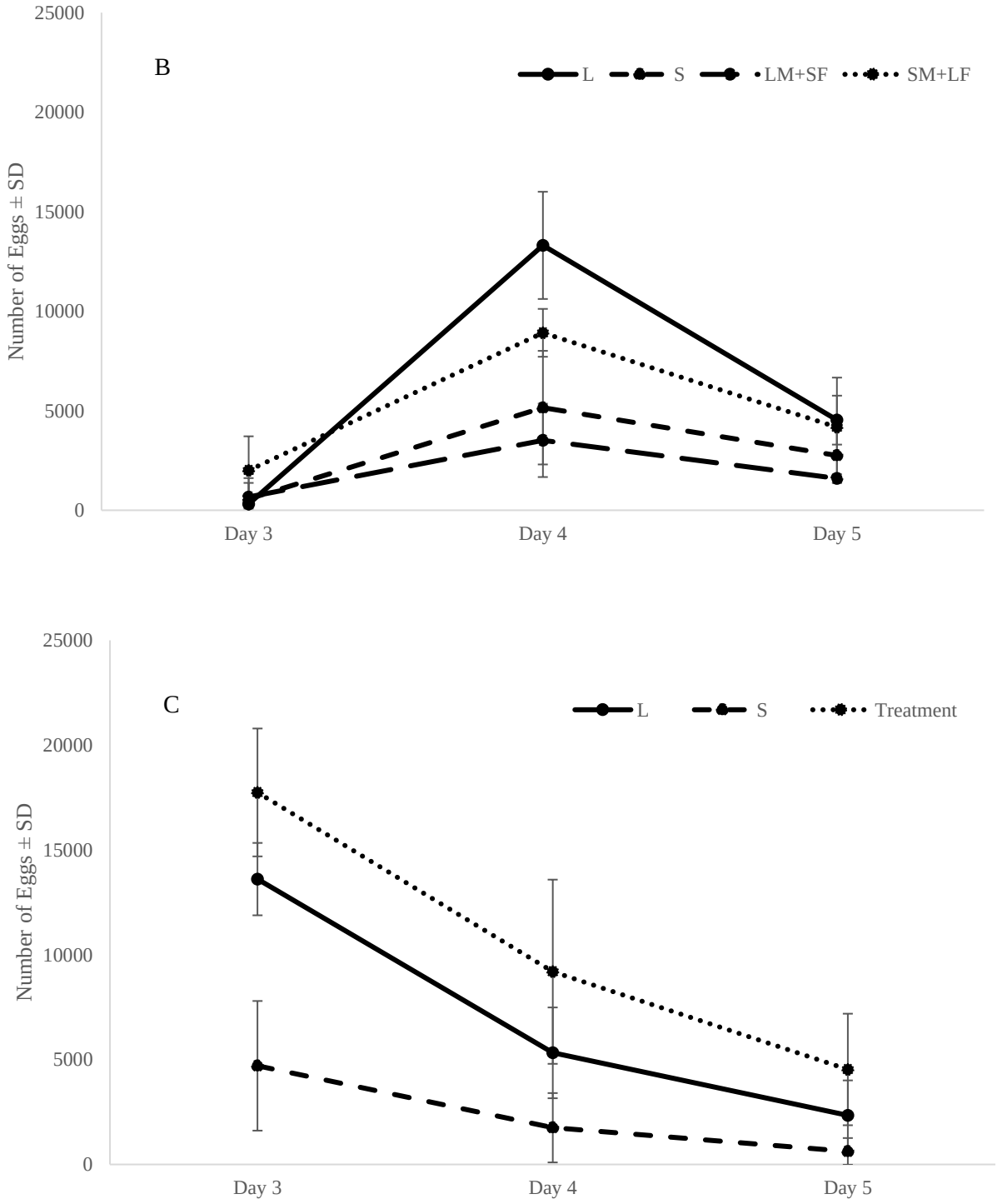
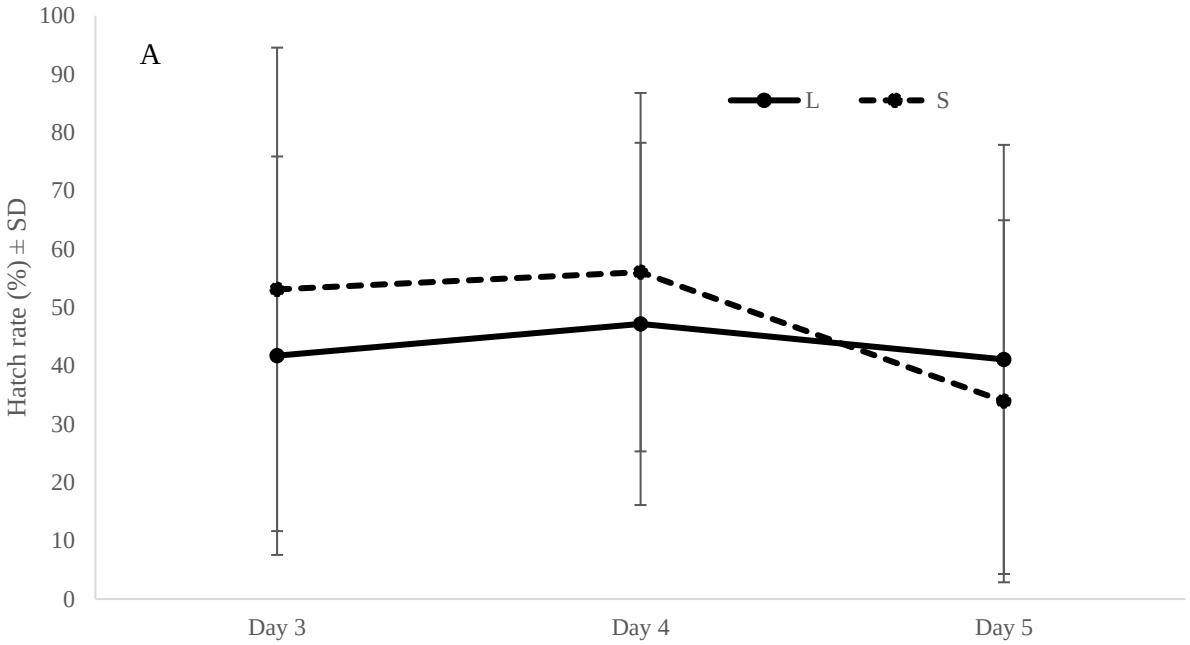


Supplementary Figure A.5: Mean hatch rate (%) ± SD (n = 4) from eggs collected consecutively after second day of mating observation A) homogenous populations (n = 4); B) 50% heterogenous populations compared to the homogenous population (control) (n = 6); and C) 25% heterogenous populations compared to the homogenous population (control) (n = 6) held in 128 x 84 x 84 cm mesh cages in a greenhouse maintained at 39 ± 4.1 °C and 44 ± 3.8% RH. L = large adults, S = small adults, LM = large males, SM = small males, LF = large females, SF = small females.



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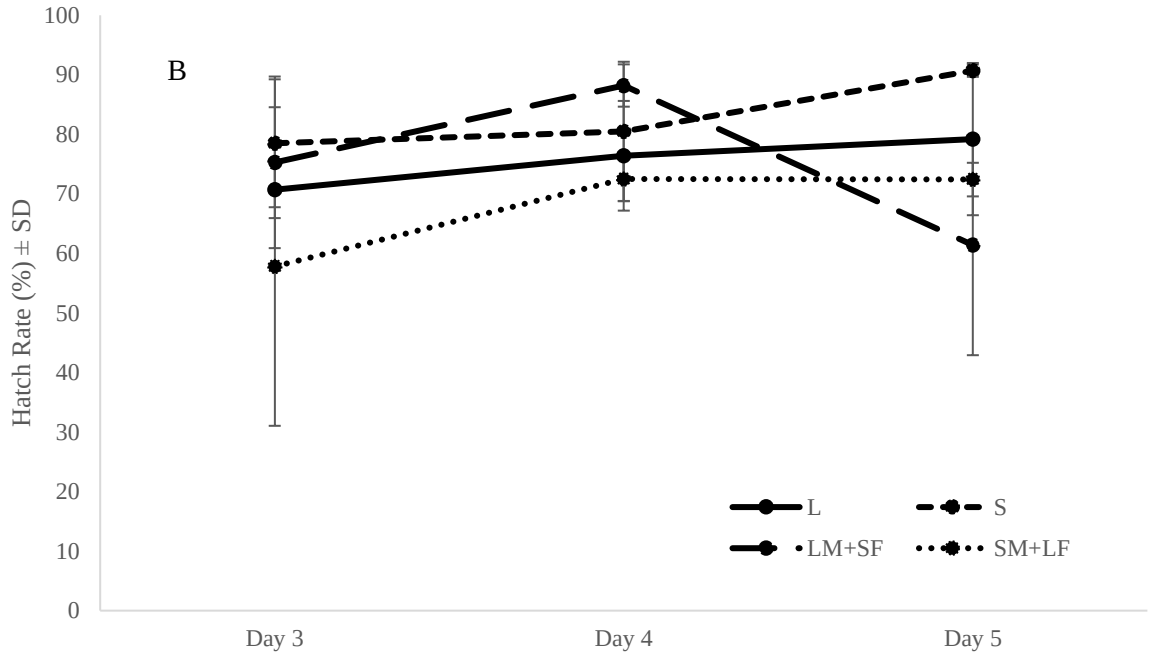
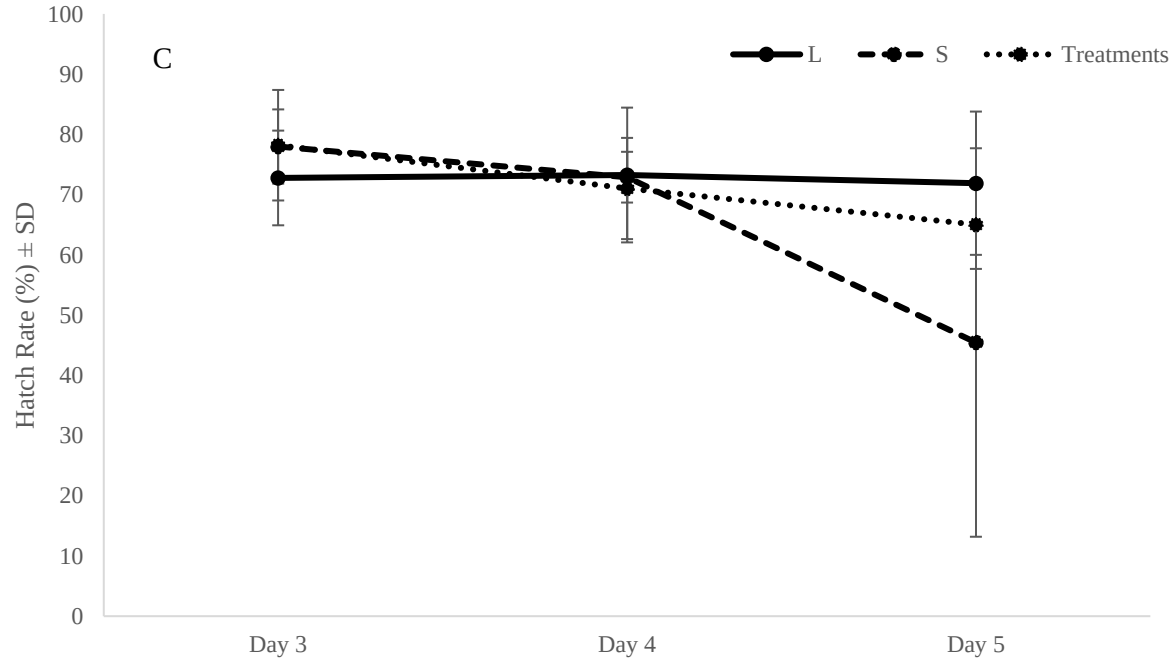




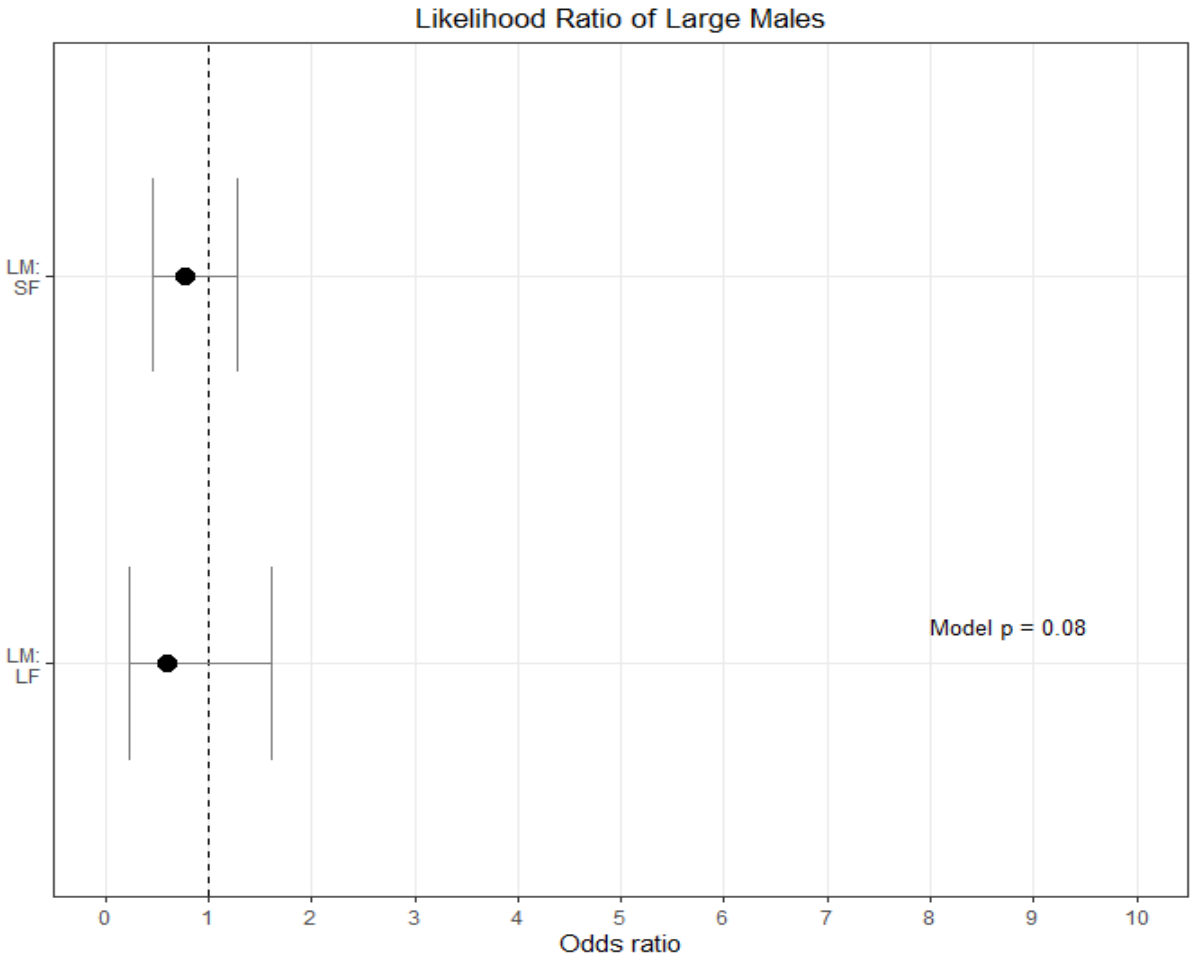
Supplementary Table A.1: Mean life-history traits ± SD (n = 6) of large and small black soldier fly adults for heterogenous (25%) populations (n = 4) compared to the homogenous population (control) where 25% of large and small adults are held in 128 x 84 x 84 cm mesh cages in a greenhouse maintained at 40 ± 1.1 °C and 50 ± 3.2% RH. L = large adults, S = small adults, LM = large males, SM = small males, LF = large females, SF = small females.

	25% (mean ± SD)								
	L	S	LM+ SF	SM+ LF	LM+ LF	SM+ SF	LM+ LM[1]	LM+SM[2]	SM+SM[3]
Successful Matings	31.00 ± 3.68 A[4]	20.00 ± 1.63 B	24.00 ± 7.13 A	9.00 ± 2.96 B	19.00 ± 1.14 B	24.00 ± 2.76 A	--	--	--
Failed Matings	8.00 ± 5.09 A	5.00 ± 2.05 A	9.00 ± 2.16 A	8.00 ± 1.69 A	7.00 ± 2.62 A	7.00 ± 1.24 A	--	--	--
Multiple Matings	3.00 ± 0.81 a[5]	2.00 ± 1.24 a	1.00 ± 0.47 b	2.00 ± 0.81 a	3.00 ± 0.47 a	1.00 ± 0.47 b	--	--	--
Male-Male Aggression	14.00 ± 2.16 A	7.00 ± 2.4 C	--	--	--	--	12.00 ± 4.78 B	6.00 ± 1.69 C	5.00 ± 2.82 D
# of Eggs	21276.00 ± 1962.67 a	7082.00 ± 4005.61 b	31384.00 ± 1898.65 c						
Hatch Rate (%)	72.63 ± 0.56 a	65.43 ± 14.29 a	71.54 ± 19.96 a						

[1] Large males with large males
[2] Large males with small males
[3] Small males with small males
[4] Different capital letters indicate significance ($p < 0.05$) between sizes from Tukey HSD analysis.
[5] Different lowercase letters indicate significance ($p < 0.05$) from Dunn post-hoc analysis

Supplementary Figure A.6: Likelihood ratio of A) large males (LM) obtaining large and small females (LF, SF respectively), and B) small males (SM) obtaining large and small females in 25% heterogenous populations compared to the homogenous population (control). Observations were done in 128 x 84 x 84 cm cages in a greenhouse maintained at 40 ± 1.1 °C and 50 ± 3.2% RH.

A)



Supplementary Figure A.6 Continued

B)