

IMPACT OF ADULT AGE ON THE REPRODUCTIVE OUTPUT OF THE BLACK  
SOLDIER FLY, *HERMETIA ILLUCENS* (L.) (DIPTERA: STRATIOMYIDAE)

A Thesis

by

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## ABSTRACT

The primary strategy for colony maintenance of the black soldier fly (BSF), *Hermetia illucens*, tends to be a continual release program in which newly emerged adult BSF are consistently introduced into a breeding cage with older individuals. Four treatments consisting of an equal sex ratio of the following combinations were used: 2-d-old males with 2-d-old females (YMYF); 6-d-old males with 2-d-old females (OMYF); 2-d-old males with 6-d-old females (YMOF); 6-d-old males with 6-d-old females (OMOF). Data collected include number of total mating and ovipositing events observed, eggs collected, and hatch rate, as well as distribution of each over time. YMYF having 2.2X more observed mating than YMOF and 2.7X more observed mating than OMOF. Approximately 97.8% of all mating observed on days 1-5. Age was not a significant factor for total number of oviposition observations. Adult age was not a significant factor for total number of eggs produced.

## DEDICATION

I dedicate this thesis to my dad, Mark Dickerson. You taught me to never leave any stone unturned, in the backyard and in life. That even the small creatures most people think are “gross” are actually beautiful and worthy of wonder and appreciation. I can still smell ladybug nests before I can see them, and our frequent hikes in the flume throughout my childhood will always be ones remembered with much happiness and gratitude. Any saturniid I have seen reminds me of the ones you carefully packed in your lunchbox to bring home to me from your logging trips when I was young. Thank you for being my adventure buddy and the reason that anytime someone asks me “Why bugs?” I say proudly that I was raised outside. Thank you for being my first best friend, and for encouraging me to run head-first into becoming the stubborn and obnoxiously curious naturalist and scientist that I am today.

Love you, Pops.

Love,

Aims

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## NOMENCLATURE

BSF	Black soldier fly
BSFL	Black soldier fly larvae
d	Day(s)
h	Hour(s)
L:D	Light:Dark cycle
RH	Relative humidity
OMOF	Old male old female
OMYF	Old male young female
YMOF	Young male old female
YMYF	Young male old female

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# CHAPTER I

## INTRODUCTION AND LITERATURE REVIEW

### **1.1 Overview**

The black soldier fly (BSF) *Hermetia illucens* (L.) (Diptera: Stratiomyidae) is being used to recycle organic wastes, [1-2], including food [3] and livestock (e.g., poultry, swine, and cattle) manure [4]. Use of BSF to recycle organic waste streams has been proven to be highly beneficial. The end results of this process are sustainably sourced protein for fish [5-6], swine [6], poultry [6-7], and people [8], biofuel production [9], significant (94-100%) reduction of pest organisms like in the case of the house fly, *Musca domestica* (L.) (Diptera: Muscidae) [10], and a reduced carbon footprint [11-13]. However, optimized production of the BSF is reliant on limited information associated with adult biology and associated fertile egg production. Obviously, without fertile eggs, larvae cannot be produced to digest the waste stream of interest. To date, no information is available on the impact of BSF adult age on the mating frequency, oviposition frequency, egg production, and associated hatch rate.

### **1.2 Introduction**

Securing a mate is a mandatory step for propagation of species reliant on sexual reproduction [14]. Clearly, for those species in question, failure to do so results in zero gene flow of that individual. Because propagation is crucial for an individual, natural selection on traits resulting in greater mating success is quite intense. One important factor of mating success is the age of the male and female engaged.

### **1.3 Age impacts mating success**

Mating success (i.e., securing a mate via copulation) is partially regulated by age for both males and females [15-19]. In fact, age impacts a number of facets of mating (e.g., fertility, acoustic signaling, and experience). This can be particularly important for some species where male ornamentation, which may play a major role in mating success, develops as they age [20]. Additional traits impacted by age include sperm quality [21-23], pheromone production [24], and courtship behavior [25].

Acoustic signals used for mate-calling, as an example, change as an animal ages and can improve mating success. Female field crickets, *Gryllus campestris* (Linnaeus 1758) (Orthoptera: Gryllidae), prefer to mate (i.e.  $\leq 72.5\%$ ) with old males (e.g. 30-d-old) that project a lowered song frequency (song frequency lowers as pegs of the stridulatory combs are worn or broken and is indicative of age) [26-27]. However, signs of aging in acoustic mate-calling are not universally attractive to females and are known to vary across species. Females may discriminate against males with worn or broken pegs, as determined for the tettigoniid bush cricket, *Ephippiger ephippiger* (Berthold 1827) (Orthoptera: Tettigoniidae) [28]. Examination of another acoustic mate-calling species with age-dependent signaling quality yielded inconclusive results due to contradictions between laboratory and field data [29]. These examples support the notion that age affects mating success with complexity and should be considered in further detail.

#### 1.3.1 Male age and mating success

Many studies have found male age to be closely related to the likelihood of securing a mate. Mating strategies vary across taxa whether females favor young males [18, 28, 30], intermediate-

aged males [14, 31-32], or old males [33-36]. However, it should be noted female selectivity in mate choice decreases as females age in many cases [30, 37-38].

#### 1.3.1.1 Female preference for young males

There are some species in which female mate choice favors young males. When choice tests were used to assess mating preference of female Mediterranean fruit flies, *Ceratitidis capitata* (Macleay 1829) (Diptera: Tephritidae), aging negatively affected the ability of old (i.e., 50-d-old) males to be selected over young (i.e., 20-d-old) males, with old males only mating 10% of the time [18]. Another study examining female medfly mate age preference found supporting evidence of female preference for young males within the species; however, there were no apparent fitness benefits gained [30]. Similarly, female *Dendrolimus punctatus* (Walker 1855) (Lepidoptera: Lasiocampidae) significantly ( $P < 0.001$ ) preferred younger (0-2-d-old) males to old (4-d-old) males with no evident fitness benefits acquired [39].

#### 1.3.1.2 Female preference for intermediate-aged males

Females are also known to favor intermediate aged males. Such a preference is not uncommon. Examples range from flies to beetles and moths; however, vertebrate examples, such as birds [40], have been documented as well. In the case of flies, Jones et al [31] determined *Lutzomyia longipalpis* (França, 1920) (Diptera: Psychodidae) female choice was heavily (i.e., 50%) skewed to favor intermediate aged males. They hypothesized younger males were unable to produce the necessary pheromones to attract females [31]. In the case of beetles, female *Colaphellus bowringi* (Baly, 1865) (Coleoptera: Chrysomelidae) that selected mates of

intermediate age experienced greater fertility [32]. Similar results were determined for the moth *Ostrinia scapulalis* (Walker, 1859) (Lepidoptera: Crambidae). Intermediate aged males completed the most successful copulations when compared against young and old male moths, but this was believed to have been a result of an increased number of copulation attempts rather than female choice [41]. Though the above listed examples of evidence favoring intermediate aged males within mating systems, there is also evidence to support older males as the female choice champion in a wide array of studies.

#### 1.3.1.3 Female preference for old males

Much theory surrounds female mate choice favoring old males as superior mates [34-35, 42]. In the case of the fruit fly *Drosophila pseudoobscura* (Fallén 1823) (Diptera: Drosophilidae), females selectively mated with old males and gained the fitness benefit of higher fecundity [36]. A study of *Drosophila subobscura* (Séguy 1938) (Diptera: Drosophilidae) determined old males achieved successful copulations much faster than younger males (e.g., a mean of 14 min sooner) and finished copulation more quickly (average of 62 s sooner) than younger males, and females mated with old males produced significantly more offspring [33]. Although it is argued that females should choose old males based on their ability to survive (i.e., presumed genetic superiority for having survived), combined with the factor of increased parental investment offered by older males, assessing female mate choice should also be done through a life-history lens (i.e., acknowledge there are trade offs between survival of an individual and their reproductive efforts) [42]. However, mate choice is not restricted to females alone.



### 1.3.2 Female age and mating success

There are several examples in nature in which males exhibit mate choice, typically promoted by evolutionary conditions such as increased male paternal investment and variation in female mate quality [86]. Although it has not been documented as extensively as male age and mating success, female age has been examined as a factor impacting male mate choice [15-16, 43]. Mate choice of male *C. capitata* determined young (10-15-d-old) females were preferred over old (35-40-d-old) females 61% of the time [43]. Based on copulation duration, *Drosophila pseudoobscura* males were also recorded to exhibit a preference for young (4-d-old) females rather than old (11-d-old) females [16]. However, only intermediate aged (8-15-d-old) males maintained this pattern of behavior [16]. Similarly, male *Drosophila bipectinata* (Duda 1923) (Diptera: Drosophilidae) preferentially mated with intermediate aged (17-18-d-old) females 82% of the time in choice tests which included young (2-3-d-old) females, and 77% of the time in choice tests which included old (32-33-d-old) females [15].

Parental investment in mating systems is more complex than mate choice based on age alone. Mating preferences for younger to older mates may shift as organisms age and gain experience [16, 44-49], proving that the interaction between males and females is one that is fluid within mating systems [16, 45] and is context-dependent [19, 45, 50]. Clearly, the mosaic of elements contributing to mating success includes abiotic and biotic considerations.

## **1.4 Overview of abiotic and biotic factors affecting mating success**

Many biotic and abiotic factors impact mating success. Described here will be a few of the main contributors from each of the abiotic and biotic categories. **Table 1.1** provides a more extensive (but not exhaustive) list.

### **1.4.1 Abiotic factors impact mating success**

Abiotic factors which impact mating success include things such as temperature, humidity, and light intensity. Small-bodied animals are known to be sensitive to microclimates within their environment, and it is intuitive that such animals would be impacted developmentally and therefore reproductively by temperature and humidity [51-52]. Light duration and intensity have been known to play a major role in mating success. For example, BSF have been observed to rely upon specific light intensities (light intensity range of 63-200  $\mu\text{mol m}^{-2}\text{s}^{-1}$  is required for mating, with peak mating success recorded above 200  $\mu\text{mol m}^{-2}\text{s}^{-1}$ ) in nature [53, but see 54] and also in indoor rearing facilities [55]. A study of the spectral sensitivity of the compound eyes of adult BSF found that the regionalization of photoreceptors in *H. illucens* are opposite of typical organization of insect photoreceptors, meaning the UV receptors of BSF are most prevalent in the ventral retina (as opposed to dorsal retina) [55]. This is believed to be of high importance for how BSF perceive conspecifics based on their spatial relation to other individuals [55], which likely has a profound impact on mating behavior.

Diet is also a worthwhile consideration when examining abiotic factors as they relate to mating success. A study conducted in 2018 found that BSF larvae which were provided diets with ~45% crude protein (e.g., brewers' yeast) resulted in adult females which produced the greatest number of eggs within the scope of the study [56]. For adult BSF, adults have historically been

considered to be non-feeding [57], but new information has shown that while feeding is not a crucial component of adult behavior, adult BSF do have a functional digestive tract [58-59]. Studies comparing reproductive output of adult flies given diets of a protein source, sugar water, or providing plain water have found offering a protein source to adult BSF can result in an increase in egg production [58] and offering sugar water can lead to increased longevity of adult flies [58-59], but water alone is most crucial for adult BSF and is the only provision required for successful reproduction [57, 59].

#### 1.4.2 Biotic factors impact mating success

There are many biotic factors that influence mating success as well, with competition and experience being two of the most documented examples. Female-female competition has been documented in some mating systems, as is exhibited by the family of giant water bugs (Hemiptera: Belostomatidae) [60-61]. In many instances, males will compete with one another for females when females are the sex with the greatest parental investment or are the limiting sex [60]. Cases in which males increase their investment beyond gametic transfer (i.e., by providing parental care) or are less abundant than females often result in a role-reversal in which female-female competition will ensue [60]. Competition in sexual selection is often related to parental investment and may manifest in a variety of ways across mating systems [45, 62]. Sperm competition (e.g., when spermatozoa from multiple males compete to fertilize the same gametes within a female's reproductive tract) is a type of male-male competition which commonly occurs among species known to mate-multiply, as is the case for the soldier fly species *Merosargus cingulatus* (Schiner 1868) (Diptera: Stratiomyidae) [63]. Males of *M. cingulatus* are known to have a plastic behavioral response to sperm competition, and male density experiments found that fertilization success was

higher (i.e., average increase of 14.4%) when males experienced high density treatments (e.g., four males present) in comparison to low density treatments (e.g., one male present) [63]. Similar findings have been documented for the Mediterranean flour moth, *Ephestia kuehniella* (Zeller 1879) (Lepidoptera: Pyralidae) [64], in which case males that were exposed to rival cues before mating produced significantly ( $p < 0.0001$ ) more eupyrene sperm (meaning true sperm containing a nucleus; whereas apyrene sperm also produced by Lepidoptera lacks a nucleus) over the course of their lifetime than control males who were not exposed to rival cues [64].

Experience is another quality which may determine how successful an individual is at obtaining successful copulations. It should be acknowledged that the effect of experience on mating success may have contrasting implications depending on whether the result is from the male or female perspective, and may also condition the individuals to exhibit some type of associative learning response to specific phenotypes based on previous experiences [45-46]. Within polyandrous mating systems, there are abundant examples of males reducing mating latency as they gain experience [44, 46]. However, female mating experience may produce a variety of results ranging from increased receptivity to multiple mates when mated with inexperienced males [49] to decreased receptivity as a learned response to sexual conflict from previous copulations [46, 62, 65].

Sexual conflict is defined as the conflict that occurs when the evolutionary interests of the two sexes are at odds [62]. Sexual conflict is costly for both sexes and results in an evolutionary arms race of sorts where each sex is co-evolving to overcome the costs imposed by the other [17, 45-46, 62, 65]. Having a large body size, as an example, can be advantageous for individuals seeking copulation [17, 45]. Males of larger body size may be able to better achieve coercive mating, or females of larger body size may be better equipped to fend off mating advances from

males attempting to copulate [17, 45]. In a recent study of BSF, it was determined that large males ( $0.04 \pm 0.01$  g) were likely able to overcome female aversion when paired with small females ( $0.03 \pm 0.01$  g) and achieved the greatest number of successful copulations of all the trials [66].

Size variation is not solely a mechanism of sexual conflict in insect reproduction, however. In the same study of BSF body size and mating behavior, it was determined that large males fertilized more eggs than small males, and that large females produced more eggs than small females [66]. This was elucidated by comparing hatch rates of egg clutches produced in the study, where homogeneous populations experienced the greatest overall hatch rate ( $84.87 \pm 3.65\%$ ), and small males mated with large females experienced the least overall hatch rate ( $68.51 \pm 2.66\%$ ) [66].

**Table 1.1A:** Compilation of abiotic factors and their effect on mating success.

<b>Abiotic Factors</b>	<b>Impact on Mating Success</b>	<b>Corresponding Literature</b>
Diet	Nutritional intake status of an individual can have a positive or negative effect on that individual's ability or likelihood of achieving mating success; Supplemental protein during adult stage can positively impact female egg production; Water availability is an essential component for some organisms to achieve meaningful (i.e., viable gametic transfer) copulation; Larval diet quality can impact female egg production	[19, 56-59]
Environmental disturbance	Environmental disturbances (i.e., auditory and light pollution, landscape restructuring) can alter how an animal emits signals, understands signals, and how those signals reach the intended recipient, which can reshape the fitness of an ecosystem	[67]
Humidity and Temperature	Humidity and temperature can be forms of selective pressure; Individuals sensitive to a given RH or temperature range will tend to have decreased mating success in that environment	[21, 51-52, 68]
Light	Specific lighting (natural and artificial) may be required to stimulate mating behavior	[53-55]
Nuptial gifts	Choosers may accept or reject mating attempts from courters with desirable or inferior nuptial gifts, respectively	[41, 45, 69]

**Table 1.1B:** Compilation of biotic factors and their effect on mating success.

<b>Biotic Factors</b>	<b>Impact on Mating Success</b>	<b>Corresponding Literature</b>
Age	Choosers may increase or decrease receptivity to courters based on courter age; Choosiness tends to decrease with female age; Aging may lead to production or reduction of attractive pheromones	[15-20, 25-26, 28-39, 41-42, 44, 46, 48]
Aggression	Aggression may lead to mating success in the immediate future, but can decrease the fitness of an individual by driving all other potential mates away	[70]
Body size	Larger females may be assessed as more fecund and thus more attractive to males; Large males may produce more attractive signals to females; Large males may better overcome female resistance	[66, 71-72]
Cuticular hydrocarbons (CHC)	CHCs can be indicative of the sexual maturation and mate quality of an individual; CHCs are often reflective of an individual's life-history traits	[73]
Competition	Competitors within a given mating system that are more successful than their rivals secure more matings (i.e., able to secure better positions on leks, able to fend off opposing courters, better suited to guard mates and other resources); Competition for developmental resources between siblings may occur, which can decrease overall growth and size, impacting future mating ability	[45, 60, 62-65, 74]
Learning/experience	Individuals with experience tend to experience increased mating success than inexperienced individuals; Experience may lead to higher-quality future matings; Experienced females may learn to defend against future unwanted matings	[44, 46, 49, 62, 65]
Microbiome	Microbes contribute to mating success or failure depending on microbe-host interactions (i.e. their effect on host behavior, physiology)	[75]
Ornamentation	Courters with superior ornamentation experience greater mating success	[20]
Parasite load	Parasitic infections can lead to decreased fitness and increased mate receptivity of host organisms	[76]
Pheromones	Individuals that are capable of producing the necessary pheromones to attract mates tend to have increased fitness	[24]
Perceived danger	Females may exhibit increased willingness to mate when perceived to be threatened	[70]

## **1.5 Objectives**

The research objectives were as follows:

1. Determine the effect of male and female age on mating success.
2. Determine the effect of male and female age on fecundity.

It should be noted that in order to construct a thorough understanding of the impact of age on the mating success and fecundity of BSF, many treatments will need to be applied in future work (see **Table 1.2**). Due to time and resource limitations, this study focused on the effect of select male (2-d-old and 6-d-old) and female ages (2-d-old and 6-d-old) with respect to mating success and fecundity.

**Table 1.2:** Outline of recommended treatments for future work.

<b>Focus</b>	<b>Treatment</b>
Control (all experiments)	2-d-old males X 2-d-old females
Male age effect on mating success and fecundity	3-d-old males X 2-d-old females
	4-d-old males X 2-d-old females
	5-d-old males X 2-d-old females
Female age effect on mating success and fecundity	3-d-old females X 2-d-old males
	4-d-old females X 2-d-old males
	5-d-old females X 2-d-old males
Interaction of male and female age effect on mating success and fecundity	3-d-old males X 3-d-old females
	4-d-old males X 3-d-old females
	5-d-old males X 3-d-old females
	3-d-old males X 4-d-old females
	4-d-old males X 4-d-old females
	5-d-old males X 4-d-old females
	3-d-old males X 5-d-old females
	4-d-old males X 5-d-old females
	5-d-old males X 5-d-old females

## 1.6 References

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## CHAPTER II

### IMPACT OF AGE ON THE REPRODUCTIVE OUTPUT OF THE BLACK SOLDIER

#### FLY, *HERMETIA ILLUCENS* (L.) (DIPTERA: STRATIOMYIDAE)

##### 2.1 Introduction

The use of black soldier fly, *Hermetia illucens*, (L.) (Diptera: Calliphoridae) larvae (BSFL) is gaining interest as an industrialized method for recycling organic waste [7, 8], including food waste [9] and livestock manure [10]. Use of BSF larvae to recycle organic waste streams has been proven to be highly beneficial. The end results of this process are sustainably sourced protein for fish [11, 12], swine [12], poultry [12, 13], and people [14], biofuel production [15], significant (94-100%) reduction of pest organisms like in the case of the house fly, *Musca domestica* (L.) (Diptera: Muscidae) [16], reducing the carbon footprint [17-19] associated with agriculture, as well as pathogen reduction, such as *Escherichia coli* and *Salmonella* [40].

With so many benefits associated with black soldier fly (BSF), it is clear why this species is agriculturally, environmentally, and economically important. However, there is little currently known about BSF mating behavior, particularly with respect to how age affects the reproductive output of BSF. A recent publication indicates increased male age was accompanied by reduced sperm viability (i.e., 144-h-old males had a 25% reduction of sperm viability compared to 48-h-old males) as well as reduced sperm quantity (i.e., 168-h-old males had 50% fewer sperm compared to 48-h-old males) [20]. This suggests there is a narrow temporal window to maximize reproductive output in the short (i.e., ~14-d) life span of male BSF. It is possible that BSF males simply do not survive long enough to exhibit a decline in sperm quality, although further study in multiple contexts will be necessary to address this uncertainty.



Protandry is defined as a mating system in which males populate a breeding site before females, either through asynchronous emergence or arrival [23, 39]. Given that BSF males generally emerge two days before females [21-22], it is necessary to explore the threshold of optimal reproductive ages for males and females because males and females may reach peak reproductive potential at different ages (e.g., days old). There may be underlying reasons (i.e., poor competitive quality or disadvantageous morphological traits) in nature which prevent old individuals from successful reproduction; however, there is currently limited research available to support this inference. Ephemeral (short-lived) protandrous insects have restricted breeding opportunities [23], therefore fitness-related pressures are applied to expedite mating success within those populations. Little is known about natural populations of BSF [24], and most available information on mating behavior of the species has been compiled via studies of captive populations [21, 25-26]. The aim of this study was to determine the impact of age of adult males and females on fertile egg production. It is critical to note here that intrinsic and extrinsic environmental factors may also significantly impact sperm quality, and the interaction between environmental factors as well as adult BSF age are context-dependent [27].

## **2.2 Methods**

### **2.2.1 Acquisition of flies**

Adult flies used in the experiments were reared from eggs produced by an indoor colony at the EVO Conversion Systems, LLC facility in College Station, Texas, USA following adapted methods described in [28]. Eggs collected from the colony were taken to the Forensic Laboratory for Investigative Entomological Sciences (F.L.I.E.S.) Facility at Texas A&M University (TAMU)

in College Station, Texas, USA, where the eggs were placed in a 946 ml cup (Uline, WI, USA) with a paper towel fastened over the opening with a metal jar ring and stored in a walk-in growth chamber maintained at 30°C, 70% RH, and 14:10 L:D photoperiod. Eggs were monitored for hatch every 24 h. Once egg hatch was noted, 100 mg of neonatal larvae were transferred to a clean 454 ml cup (Uline, Wisconsin, USA), fed 100 g Gainesville diet at 70% moisture (adapted from Jones and Tomberlin, 2020) [26], covered with an upside-down 532 ml cup to reduce moisture loss, and returned to the walk-in incubator. After eight days, contents of the cup were transferred to a plastic shoebox (35.6 (L) x 20.3 (W) x 12.4 (H) cm) containing 400 g Gainesville diet at 70% moisture. Approximately 75 g of dry Gainesville diet was spread around the outer edges within the shoebox to prevent larval escape. The same 454 ml cup which previously held the larvae was used to cover the larvae on the fresh feed within the shoebox to abate moisture loss. After three days, the contents of the shoebox were placed in a plastic storage pan (33.0 (L) x 21.6 (W) x 30.5 (H) cm) containing 4 kg of Gainesville diet at 70% moisture with 1 L of dry diet placed around the inside perimeter of the pan to reduce larval escape and returned to the incubator. Once 40% of the larvae reached the prepupal stage (approximately 21 days post egg hatch), the larvae were removed using a sifting pan (33.7 (L) x 33.7 (W) x 8.9 (H) cm) with a mesh screen of 0.6 cm (Sona Enterprises, CA, USA), and distributed evenly between 1 L deli cups (Uline, WI, USA), covered with mesh fastened by a rubber band, placed in the incubator previously described, and monitored daily for adult emergence [29]. Larval cohorts were staggered within colony generation to align age groups for inclusion in the research conducted.

Adult flies were sorted at emergence based on morphology [30]. Males and females were held separately in plastic fly cages (30 (L) x 30 (W) x 30 (H) cm) (Bioquip Products, CA, USA)

[29] in the walk-in growth chamber until use. Flies in each cage were provided 30 mL water twice daily via a 3.79 L garden sprayer (Chapin Manufacturing, NY, USA).

### 2.2.2 Experiment design

Four age treatments (**Table 2.1**) each with three replicates were used in the following experiment, which was replicated twice (i.e., two generations). All treatments were comprised of an equal sex ratio. The control was set at two-day-old males and females [21-22]. Adult flies (100 male and 100 female) for a given treatment were released in an Insect-a-Hide pop-up cage (84 cm (L) x 84 cm (W) x 133 cm (H)) (Lee Valley Tools Ltd., Ottawa, Ontario, Canada) on the floor of room 9 in building 1043 of the F.L.I.E.S. Facility at TAMU maintained between 19-35°C. Cages (i.e., replicates) were arranged in a randomized design. Each cage was provided a 50-watt light-emitting diode (LED) light (HK SPR AGTECH Trading LTD, Hong Kong, China) fixed at ~101 cm high and <1 cm from the cage on a 14:10 L:D cycle. Temperature [30-31] and humidity [22, 32-33] were recorded daily within the room using a HOBO® data logger model MX1104 (Onset Computer Co., MA, USA). Each cage was misted three times daily with 100 ml of water at the beginning, middle, and end of the light cycle for the day.

An attractant box (i.e., oviposition site) was placed in each cage after the second watering at the midpoint of the light cycle on the second day of the experiment [31]. Attractant boxes consisted of plastic shoeboxes (35.6 (L) x 20.3 (W) x 12.4 (H) cm) containing 500 g Gainesville diet (70% moisture), approximately 75 g dry Gainesville diet, and approximately 1000 7-d-old BSF larvae. The lid of the shoebox was modified by replacing a 12.7 (L) x 5 (W) cm section in the center with mesh. Attractant boxes were elevated by setting them on top of two upside-down 473 mL plastic cups (Dart Container, IL, USA). An egg trap was placed onto the mesh portion of the

shoebox lid with the corrugations facing laterally. Egg traps were made of three strips of stacked corrugated cardboard held together with masking tape, with completed egg traps measuring 10 (L) x 3.5 (W) x 1.25 (H) cm [29, 32, 33]. The resulting eggs from each treatment were collected daily from each cage after LED lights were turned off and once oviposition ceased. The experiment was terminated once 24 h passed without oviposition occurring in any of the cages (i.e., zero eggs collected from the experiment in 24 h).

Eggs collected daily were removed from the cardboard traps via a size 0 round synthetic paintbrush (Daler-Rowney Ltd, Berkshire, England), placed into a 30 mL cup, and weighed using an Ohaus Scout® Pro Balance Scale (Ohaus Corporation, NJ, USA). The individual 30 mL containers then were housed in 240 mL glass jars with a paper towel fastened by tightening a metal jar ring as a lid. The jars were kept in a walk-in growth chamber previously described and allowed to hatch. After a minimum of 10 d, all jars from each replicate were assessed using a ZEISS Stemi 305 stereo microscope (ZEISS Group, Oberkochen, Germany) to determine whether any larval hatch occurred.

**Table 2.1:** Adult black soldier fly, *Hermetia illucens* (L.) (Diptera: Stratiomyidae) treatments examined in mating and oviposition experiments conducted in an indoor facility located at Texas A&M University, College Station, Texas.

<b>Focus</b>	<b>Treatment</b>	<b>Abbreviation</b>
Control (all experiments)	2-d-old males X 2-d-old females	YMYF
Male age effect on reproductive output	6-d-old males X 2-d-old females	OMYF
Female age effect on reproductive output	2-d-old males X 6-d-old females	YMOF
Interaction of male and female age effect on reproductive output	6-d-old males X 6-d-old females	OMOF

### 2.2.3 Statistical analysis

A one-way analysis of variance (ANOVA) was used to determine if age of male and female adult flies impacted total mating pairs observed, total oviposition events, and total eggs produced. A two-way ANOVA was performed to determine if age of male and female adult flies affected mating pairs, oviposition events, and eggs produced over time. All statistical analyses were performed using R<sup>®</sup> software version 4.1.2 [34]. Tukey's test was used to separate means with significance level set at  $p < 0.05$ . In cases of multiple comparisons, Bonferroni corrections were made.

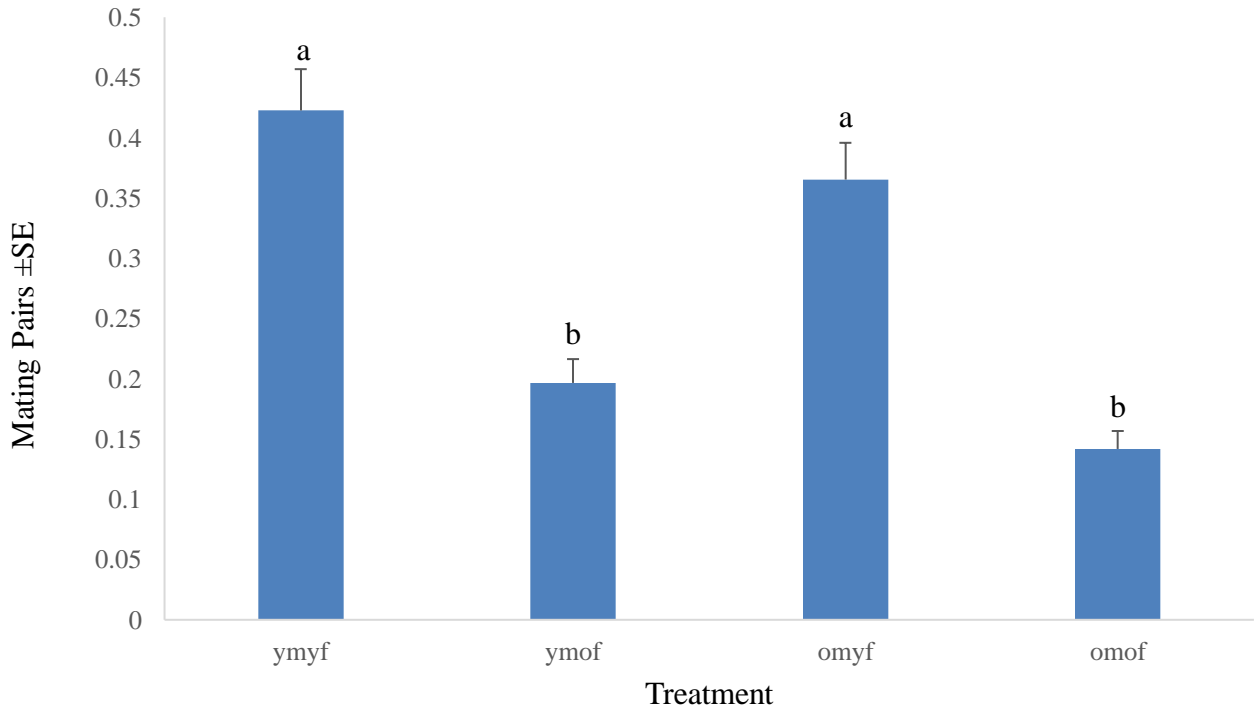
The odds ratios of oviposition events or associated egg hatch were tested and plotted using in R version 3.4.3, the DescTools package (<https://cran.r-project.org/web/packages/DescTools/index.html>). All treatments were compared to YMYF. In order to determine whether the responses were significantly different ( $p \leq 0.05$ ) across treatments and trials, the data were analyzed using a generalized linear mixed model (GLMM) for a binomial distribution with the logit link-function to model probabilities.

## **2.3 Results**

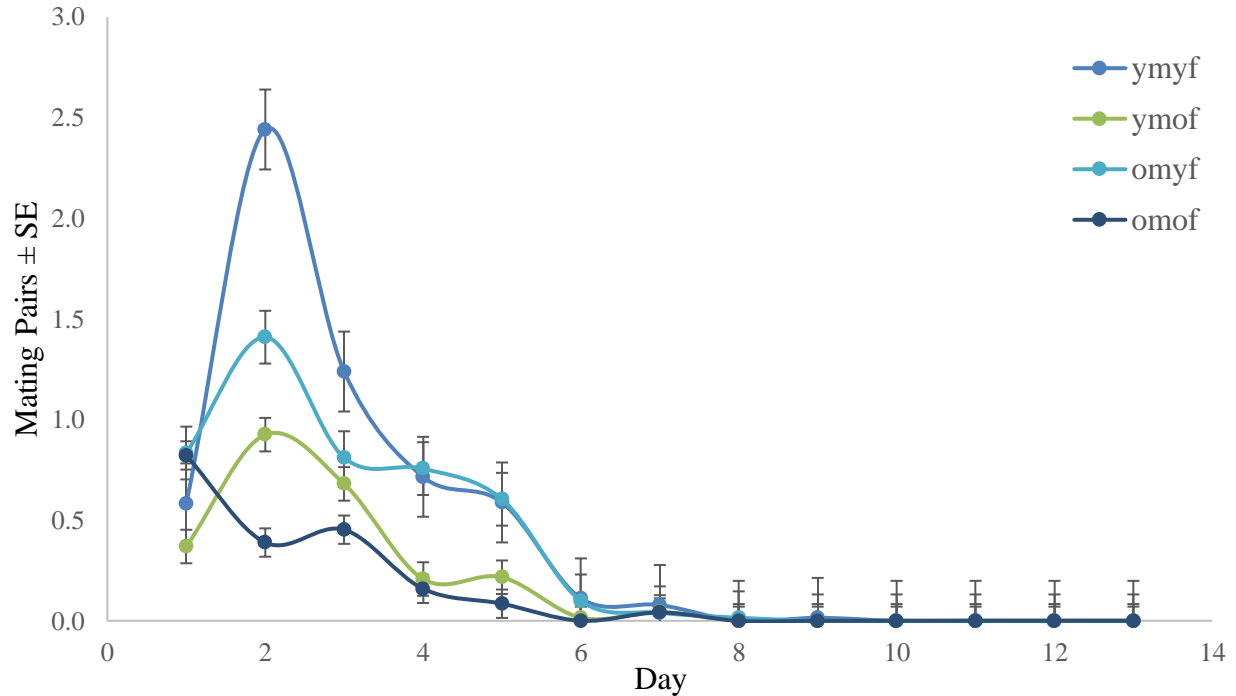
### 2.3.1 Mating observations

Adult age significantly ( $F_{3,4199} = 37.70$ ,  $p < 0.001$ ) impacted number of mating events (**Figure 2.1**). Pairwise comparison with Bonferroni corrections indicated significant differences between YMYF x OMOF ( $p < 0.001$ ) and YMYF x YMOF ( $p < 0.001$ ). The YMYF combination had 3.1X more total mating events than the OMOF combination, and 2.4X more than YMOF combination. When the model was expanded to include time as a factor, the interaction between

day and number of mating observations per treatment was significant ( $F_{36,4199} = 10.24, p < 0.001$ ) (Figure 2.2). Mating pairs for the YMYF combination on Day 2 were 6.4X greater than for the OMOF on the same day. 97.5% of total mating were recorded on days 1-5. Mating activity for all treatments ceased after Day 9.



**Figure 2.1:** Mean number of observed mating pairs  $\pm$  SE for BSF mating experiments at  $\sim 26^{\circ}\text{C}$  and  $\sim 45\%$  RH. YMYF = young male young female; YMOF = young male old female; OMYF = old male young female; OMOF = old male old female.

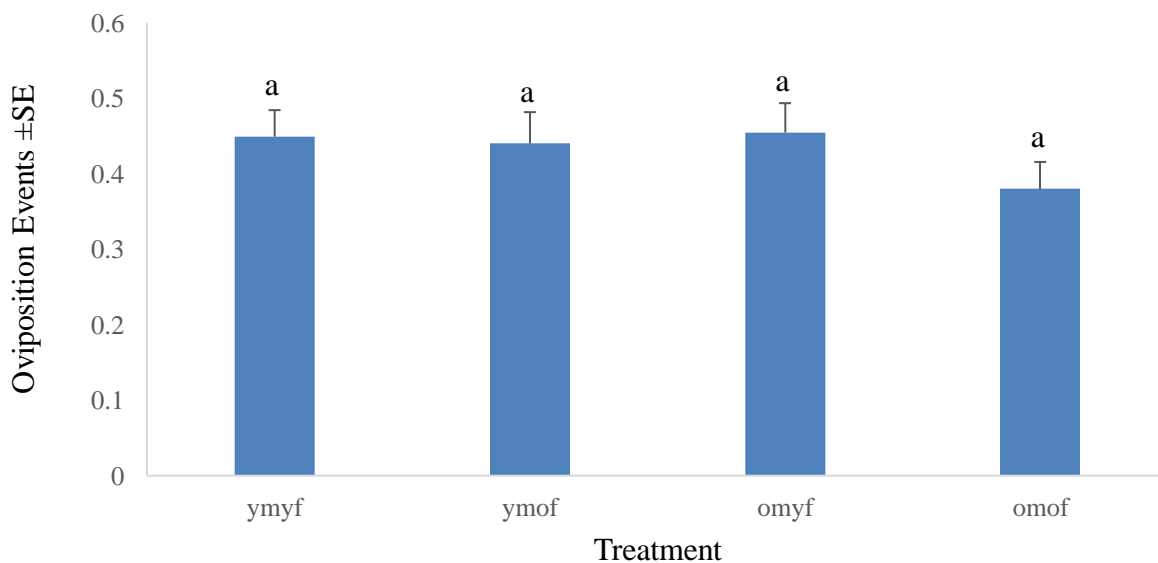


**Figure 2.2:** Mean number of observed mating pairs per day  $\pm$  SE for BSF mating experiments at  $\sim 26^{\circ}\text{C}$  and  $\sim 45\%$  RH. YMYF = young male young female; YMOF = young male old female; OMYF = old male young female; OMOF = old male old female.

### 2.3.2 Oviposition observations

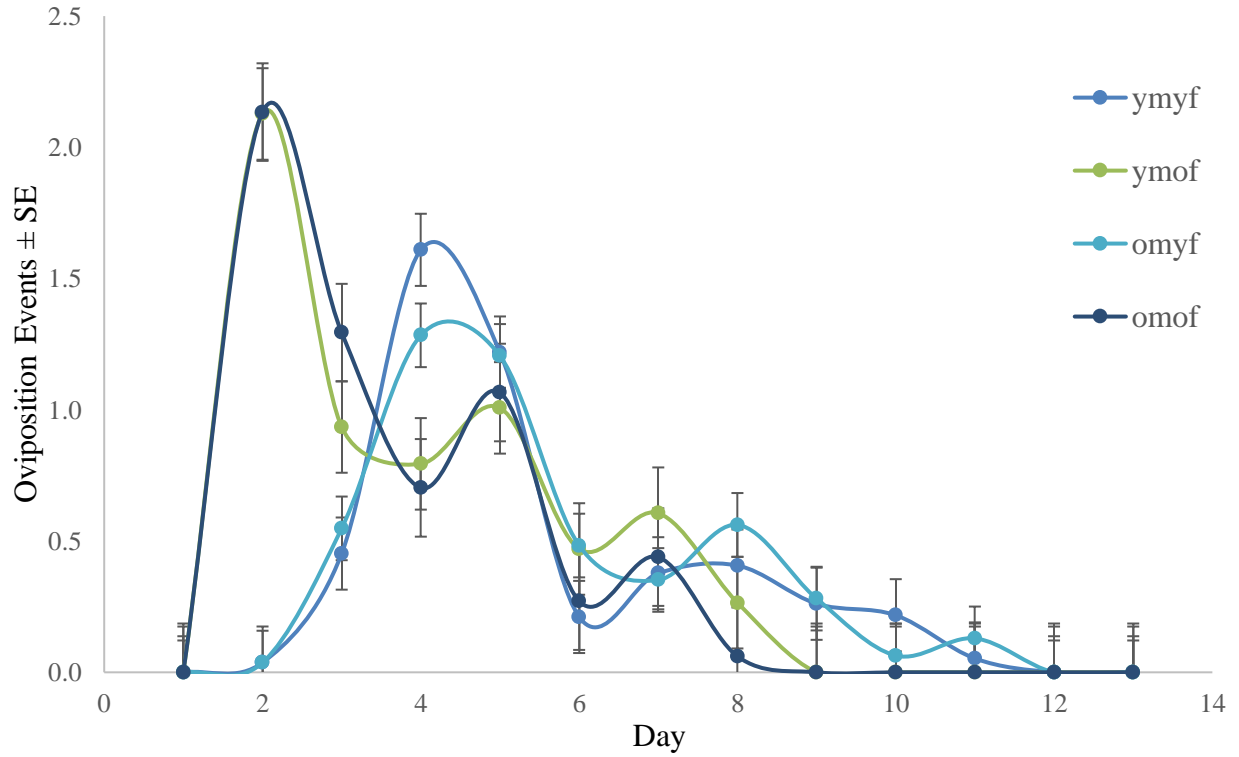
Adult age did not significantly ( $F_{3,3544} = 0.200$ ,  $p = 0.8966$ ) impact total oviposition observations by treatment (**Figure 2.3**). When the model was expanded to include time as a factor, day was the only significant ( $F_{33,3544} = 84.7$ ,  $p < 0.001$ ) variable. YMYF and OMYF oviposition observations peaked during days 4-5, while YMOF and OMOF exhibited a bimodal distribution on days 3 and 5 with a recession in observations during day 4 (**Figure 2.4**). With regards to odds ratio analysis for all data collected over the 12-d- experiment, YMOF oviposition occurring was significantly ( $p = 0.0061$ ) less likely (0.33) when compared to the YMYF. Similarly, oviposition events for OMOF compared to those recorded for YMYF were significantly ( $p < 0.0001$ ) less

likely (0.25). In contrast, the likelihood of oviposition events for OMYF was not significantly ( $p = 0.2890$ ) different than the YMYF but was less likely (0.64) (**Figure 2.5A-B**). Furthermore, the binomial GLMM analysis determined day ( $F_{1,1} = 92.88, p < 0.001$ ), treatment ( $F_{3,3} = 8.08, p < 0.001$ ), and the day by treatment interaction ( $F_{3,3} = 23.84, p < 0.001$ ) were significant. Replicate ( $F_{1,1} = 0.102, p = 0.749$ ), as well as remaining interactions were not significant. However, when focused within a subset of days (e.g., 2-6-d) during the experiment, the number of oviposition observations were greater than those for days 7-12 for all treatments, ranging from 3.5X greater for both YMYF and OMYF, 11.4X greater for YMOF, and 17.9X greater for OMOF. 85.0% of the total oviposition observations were recorded from days 2-6. Oviposition activity for all treatments ceased after day 12.



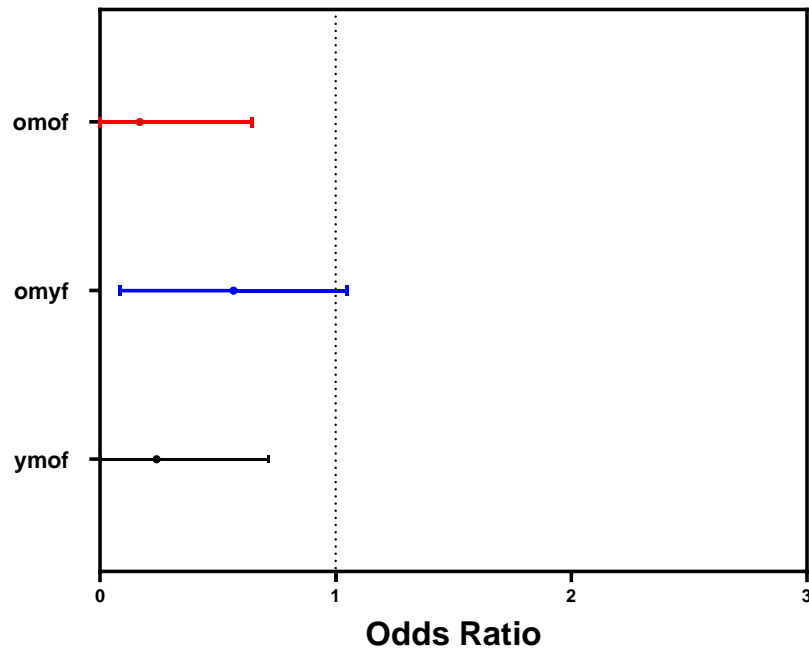
**Figure 2.3:** Mean number of observed oviposition events  $\pm$  SE for BSF mating experiments at  $\sim 26^{\circ}\text{C}$  and  $\sim 45\%$  RH. YMYF = young male young female; YMOF = young male old female; OMYF = old male young female; OMOF = old male old female.





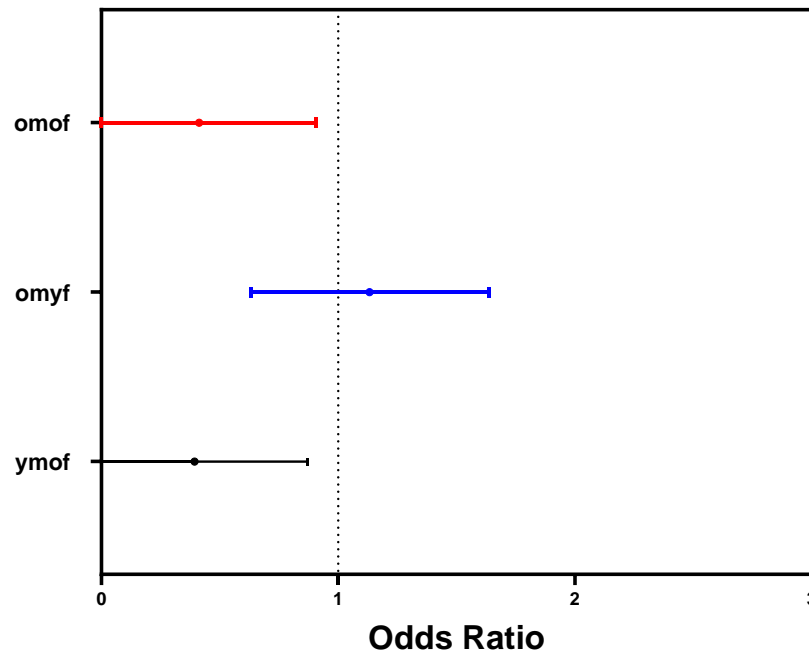
**Figure 2.4:** Mean number of observed oviposition events per day  $\pm$  SE for BSF mating experiments at  $\sim 26^{\circ}\text{C}$  and  $\sim 45\%$  RH. YMYF = young male young female; YMOF = young male old female; OMYF = old male young female; OMOF = old male old female.

(2.5A)



**Figure 2.5A:** The odds ratio for oviposition events recorded from BSF mating experiments versus YMYF = young male young female (control); YMOF = young male old female; OMYF = old male young female; OMOF = old male old female. BSF mating experiments were conducted at  $\sim 26^{\circ}\text{C}$  and  $\sim 45\%$  RH.

(2.5B)

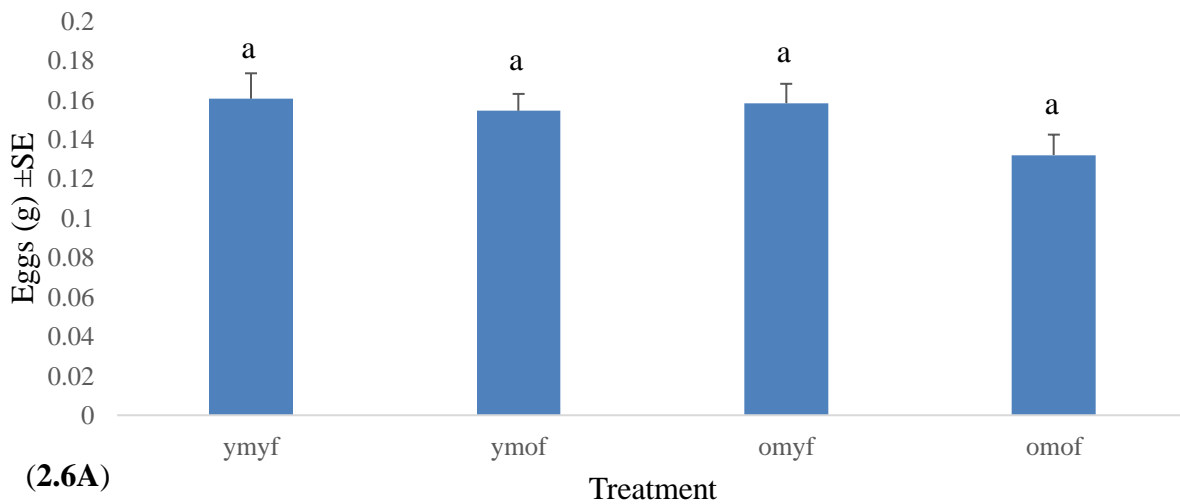


**Figure 2.5B:** The odds ratio for hatch for eggs collected from BSF mating experiments versus YMYF = young male young female (control); YMOF = young male old female; OMYF = old male young female; OMOF = old male old female. BSF mating experiments were conducted at ~26°C and ~45% RH.

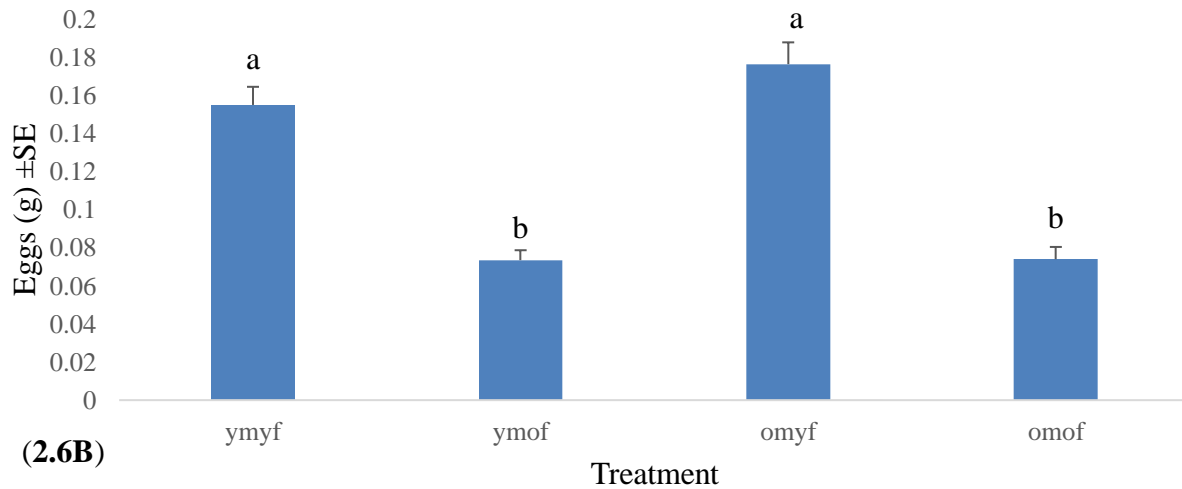
### 2.3.3 Egg Production

Age of adult flies significantly ( $F_{3,3856} = 19.18, p < 0.001$ ) impacted number of total eggs (**Figure 2.6A-B**). There was a significant trial effect ( $F_{1,3856} = 22.14, p < 0.001$ ) (**Figure 2.6A-B**). A GLM was performed to assess occurrence of a trial by treatment interaction, which was found to be significant ( $p < 0.001$ ). When the model was expanded to include time as a factor, day was found to be significant ( $F_{33,3772} = 435.93, p < 0.001$ ) (**Figure 2.7A-B**). When focused within a subset of days (e.g., 2-6-d) during the experiment, the number of eggs collected for days 2-6 were

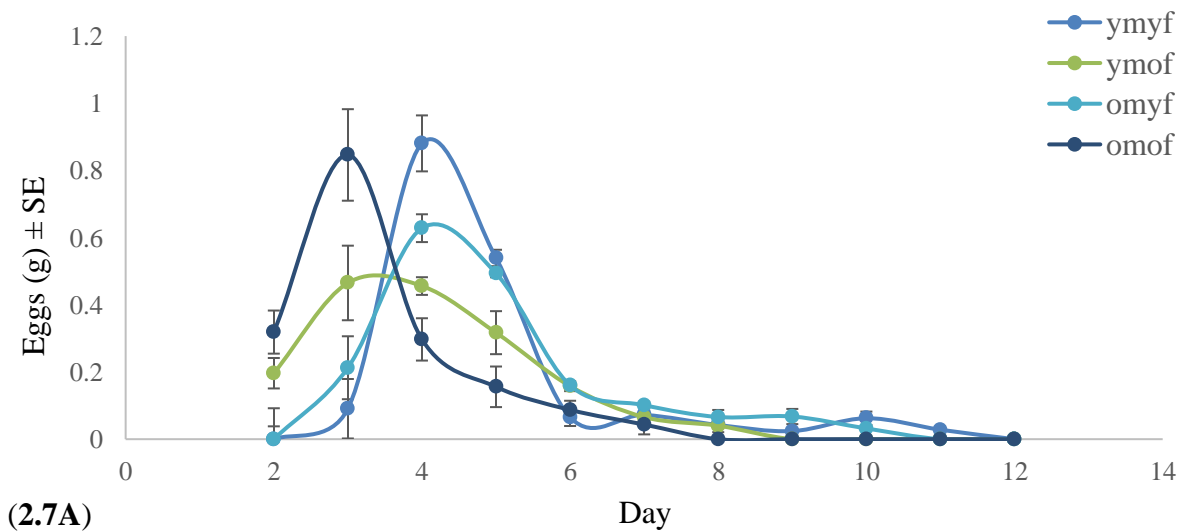
greater than those for days 7-12 for all treatments, ranging from 13.1X greater for YMYF, 9.4X greater for OMYF, 31.7X greater for YMOF, and 108.1X greater for OMOF. **Figure 2.7A-B** indicates treatments with old females (YMOF and OMOF) oviposited a day earlier than treatments with young females (YMYF and OMYF). Further, all treatments with old females reached peak daily oviposition on day 3, and all treatments with young females reached peak daily oviposition on day 4 (**Figure 2.7A-B**). For total eggs deposited, 94.8% were collected from days 2-6. Oviposition activity for all treatments ceased by day 12.



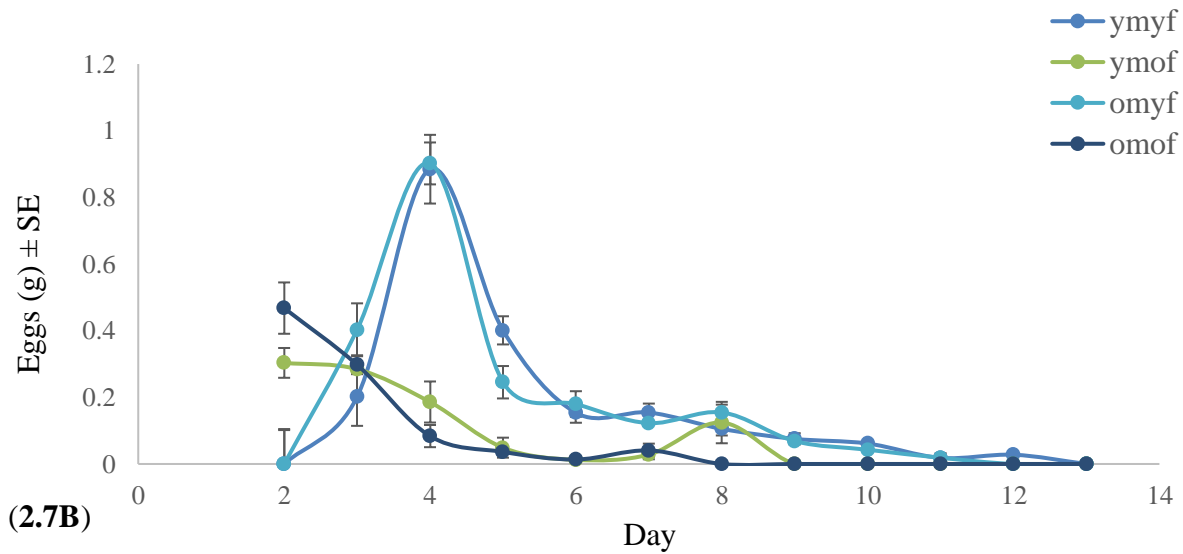
**Figure 2.6A:** Mean eggs (g) collected  $\pm$  SE from Trial 1 for BSF mating experiments at  $\sim 26^{\circ}\text{C}$  and  $\sim 45\%$  RH. YMYF = young male young female; YMOF = young male old female; OMYF = old male young female; OMOF = old male old female.



**Figure 2.6B:** Mean eggs (g) collected  $\pm$  SE from Trial 2 for BSF mating experiments at  $\sim 26^{\circ}\text{C}$  and  $\sim 45\%$  RH. YMYF = young male young female; YMOF = young male old female; OMYF = old male young female; OMOF = old male old female.



**Figure 2.7A:** Mean eggs (g) collected  $\pm$  SE per day for BSF mating experiments from Trial 1 at  $\sim 26^{\circ}\text{C}$  and  $\sim 45\%$  RH. YMYF = young male young female; YMOF = young male old female; OMYF = old male young female; OMOF = old male old female.



**Figure 2.7B:** Mean eggs (g) collected  $\pm$  SE per day for BSF mating experiments from Trial 2 at  $\sim 26^{\circ}\text{C}$  and  $\sim 45\%$  RH. YMYF = young male young female; YMOF = young male old female; OMYF = old male young female; OMOF = old male old female.

### 2.3.4 Egg hatch

No significant difference ( $p > 0.05$ ) across treatments when analyzed with an ANOVA; however, OMYF showed relatively similar hatching rate compared with the control (**Figure 2.5b**). With regards to odds ratio analysis, YMOF egg hatch was significantly ( $p = 0.0483$ ) less likely (0.39) when compared to the YMYF. Similarly, the likelihood of egg hatch for OMOF when compared to egg hatch for YMYF was, while marginal, significantly ( $p = 0.0708$ ) less likely (0.41). In contrast, OMYF was not significantly ( $p = 0.8039$ ) different from YMYF (1.13). Similar to analysis of raw count data previously presented, when comparing means categorical responses (hatch v no hatch) across treatments over time, the binomial GLMM determined the following to be significant (trial:  $F_{1,1} = 17.55$ ,  $p < 0.001$ ; day:  $F_{1,1} = 9.13$ ,  $p = 0.003$ ; trail by day:  $F_{1,1} = 9.61$ ,

$p = 0.0023$ ; treatment:  $F_{1,3} = 6.32$ ,  $p < 0.001$ ; trial by treatment:  $F_{3,3} = 8.63$ ,  $p < 0.001$ ; day by treatment:  $F_{3,3} = 3.12$ ,  $p < 0.027$ ; trial by day by treatment:  $F_{3,3} = 3.15$ ,  $p = 0.027$ ). Replicate ( $F_{1,1} = 0.24$ ,  $p = 0.621$ ), as well as remaining interactions, were not significant.

## **2.4 Discussion**

When assessing the productivity of a BSF colony, there are many surface-level factors that may not serve as adequate indicators of the reproductive utility of the colony. Such factors, as those assessed in the current research, that may be misleading include observations of mating pairs, ovipositing females, and even eggs produced. These factors occur in concert, but the crux of determining reproductive output within a colony ultimately is hatch rate.

Number of mating pairs occurring over time or total was not a suitable method for determining fertile egg production. In the case of BSF, adults in colony will mate multiple times. For example, Jones and Tomberlin [26] reported a 10% remating rate of black soldier flies in colony. Furthermore, males lose 25% of sperm viability by 6-d-old compounded by a loss of 50% of sperm cells by 7-d-old [20], thus the likelihood of fertilizing eggs is reduced as the males continue to age. When comparing to Malaway et. al (2020) [20], the adequacy of relying upon observation of mating pairs as a measure of fertile egg production becomes increasingly unsubstantiated. The present study observed peak mating behavior to occur on the second day of the study, making the age of the young males 4-d-old and the old males 8-d-old. Supplementary tables from [20] reported that 4-d-old males already had a reduced sperm viability of ~10% and a reduced sperm count of ~12%. Although the findings of Jones and Tomberlin [26], Malaway et. al 2020 [20], and the present study are not able to be explicitly transposed due to generational differences of the black soldier flies utilized within the study, it may still be inferred that

reproductive viability of adult male black soldier flies decreases as soon as 48 h post-emergence, regardless of visual indicators to the contrary (i.e., increase in number of mating pairs).

Other than when analyzed with odds ratio, raw counts of oviposition events, as well as eggs collected (over time or total), also were not informative indicators of mating success. Black soldier flies are known to lay sterile eggs [21], and this behavior has been recorded for both wild and colony populations in foundational studies, such as Tomberlin et al. 2002 [35], where 1% virgin females laid sterile eggs. This has also been reported by studies focusing on optimizing artificial light use to stimulate BSF reproduction [36]. Nakamura et al. [36] reported 11.2% of clutches produced under LED to be fertilized, and 39.5% of clutches produced under sunlight to be fertilized [36]. It was also observed during the preliminary work for this study that virgin females would oviposit in the plastic bug dorms while being held prior to use for the mating experiments. OMOF oviposition observations was greatest of all treatments on day 2 and was 23X greater than combinations containing young females (YMYF, OMYF) and 1.4X greater than YMOF (**Figure 2.4**).

Categorical assessment of hatch data indicated old females had the greatest impact on the likelihood of eggs deposited actually hatching. For example, none of the eggs collected from peak oviposition events for old females were viable. The percentage of nonviable eggs produced by old females during these peak oviposition events on day 2 made up 13% of the overall total of eggs collected from cages containing YMOF and 19.5% of the overall total of eggs collected from cages containing OMOF. In contrast, treatments containing young females were found to only have two recorded observations of oviposition behavior occurring on day 2, and no eggs were present in cages containing young females on this day. Age of adults impacts fertile egg production for BSF in colony, and there are behavioral tradeoffs that may occur due to selective pressures of varying



intensity according to the age of the organism. Interestingly, when old males were combined with old females, the number of matings on day one was the greatest of all treatments (**Figure 2.2**), which would be misleading in terms of colony success due to the large percent of eggs not hatching. This result is not surprising, as similar behaviors have been recorded for other species. Egg viability of Indianmeal moths, *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae), has been found to decrease by 22% for every day that mating was delayed [37]. Similarly, a study examining the trade-off between fecundity and longevity of *Dichelops furcatus* (Hemiptera: Pentatomidae) reported egg dumping behavior of inviable eggs during the second half of the reproductive period [38]. Regardless, these data support the necessity of distinction between number of eggs produced versus reproductive achievement.

#### 2.4.1 Industry outlook

I encourage rearing facilities to standardize all steps of the rearing and breeding process in a way that allows for specific adjustments to be made as necessary, such as larval density, larval diet, feeding regimen, abiotic rearing conditions (i.e., temperature, RH, L:D, airflow), cage densities, watering regimen for adults, abiotic conditions for breeding cages, and duration of cage life before culling. It would also serve many within the industry to keep entomologists on staff and involved in rearing and breeding protocols. It has been my experience while working in industry that BSF companies may sometimes be lacking entomologists on their team, and this is to their detriment.

Currently, many BSF-breeding facilities utilize continuous release likely due to the ease of maintenance for such a system. Eggs produced is not a sound method for determining whether a BSF breeding cage is performing well, due to the simple fact that eggs produced does not equate

to larvae hatched. Based on results from the current study, structured management of the age of adults released into a colony should be implemented with an emphasis on adults 2-4-d-old. Furthermore, efforts to monitor egg hatch rather than mating or oviposition frequency should be recorded. By gaining greater precision with regards to hatch rate, facilities can better predict colony health, waste digestion, and insect biomass production.

Further, I encourage those within the BSF industry to look to other platforms with the shared goal of mass-reared insects, such as those reared for the purposes of integrative pest management (IPM) and biocontrol. IPM and biocontrol have methodologies already in place for mass-producing insects that maintain specific desired standards, and there is much knowledge to be gained by looking to the pest control industry.

## 2.5 References

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## CHAPTER III

### SUMMARY AND CONCLUSIONS

#### 3.1 Summary of findings

My research was completed by conducting two trials. Both showed consistency of the control (young male:young female (YMYF)) and demonstrated that increased age of both male and female BSF lead to high variability in reproductive output in breeding experiments. When assessing the effect of adult senescence on mating success, YMYF had 2.2X more observed matings than young male:old female (YMOF) and 2.7X more observed matings than old male: old female (OMOF) (**Chapter 2, Figure 2.1**). Further, 97.8% of all matings were observed on days 1-5 (**Chapter 2, Figure 2.2**). Age did not significantly impact the total number of oviposition observations (**Chapter 2, Figure 2.3**), but the inclusion of time as a factor was significant with 91.5% of all oviposition observations occurred on days 2-6 (**Chapter 2, Figure 2.4**). Similarly, adult age was not a significant factor for total number of eggs produced (**Chapter 2, Figure 2.6A-B**), but the inclusion of time as a factor was significant. 94.8% of total eggs were collected on days 2-6. Based on odds ratio analysis, old females had the greatest impact with the likelihood of eggs hatching being significantly ( $p < 0.05$ ) reduced (e.g., 50%) when paired with young or old males. In the case of old males being paired with young females, while not significant, the likelihood of producing neonates was reduced by 20%. Odds ratio analyses indicated mating OF with either category of male resulted in reduced oviposition and hatch.

While the present study was intended to be conducted in a greenhouse, the work was instead completed indoors on the Texas A&M University campus due to unprecedented storms rendering the greenhouse space unusable for an extended period. This change did allow for a reduction in variability of abiotic factors, such as by providing more stable temperature and relative humidity

ranges, as well as more consistent light exposure due to the use of artificial light (rather than having interference from cloud cover and shifts in weather patterns). The treatments that were employed were chosen based upon the principle of “strong inference”- meaning to start simple and with the most extreme (but still likely) combinations [1], and then use that as a platform for expansion if necessary.

### **3.2 Rationale and application**

By having a better understanding of when most reproductive activity occurs within a BSF breeding cage (within the first week after emergence), as well as assessing the manner in which reproductive output may be lost (i.e., OF reducing the likelihood of eggs hatching by 50%, regardless of the age of the male breeding partner), industry may be able to make better-informed decisions on when to cull breeding cages to maximize hatchability for the eggs produced. The research conducted herein may potentially be used to reduce variability in reproductive output in BSF colonies, which has been a hurdle for the industry globally. In doing such, improved breeding programs can lead to increased protein production as well as more stable organic waste valorization initiatives.

### **3.3 Potential limitations**

This study only looks at one population and there are generational differences within the study. Also, BSF larval diet may have an impact on timing and reliability of reproductive output of adult BSF, and this study was completed with use of the Gainesville diet [2]. This diet may not be accessible in other localities, and it is well documented in the literature that larval nutrition



impacts adult size as well as fecundity [3-5]. Hatch rate was only able to be assessed to “hatch/no hatch” classification due to time constraints. With adequate resources, fertilization rate could be explored in further detail. Other details of this project that could be adjusted and impact the outcome may include (but are not limited to) replicating this study in an outdoor or greenhouse space or shifting the sex ratio from equal to skewed. **Table 3.1** provides a more detailed description of limitations.

**Table 3.1:** Genetics and the limitations of the proposed research.

Limitative Factors	Potential Consequences	Corresponding Literature
Genetics	The understanding of genetic diversity and hybridization of BSF is still in its infancy, and experimentation with strains that differ from the colony maintained at F.L.I.E.S. may produce alternative findings than the proposed research (i.e., by manner of interaction between the focal strain and their diet, microbial community, mediation of abiotic factors, experimental density, and experimental sex ratio).	[6-7]
Diet & Microbial community	Insect behavior is known to be affected by their microbial profile, causing shifts in insect response to things like hormone production, receptivity to mating signals, foraging behavior, and defensive behavior; The microbial community present within the TAMU colony may be population specific and could potentially yield results in mating behavior and oviposition stimulation that are unique to this population; Diet has been shown to affect insect fecundity with BSF as a specific example; Insect diet is a factor known for changing the microbial profile of insects and is reflected in CHC pheromones, which has been shown to alter mating preferences (e.g., acceptance or rejection), particularly in Dipteran species. Diets other than the formulation to be used for this study may generate alternative results.	[8-12]
Abiotic variability	While greenhouses generally provide more stable conditions than outdoor environments, there is still a great deal of abiotic variation (see <b>Table 1.1</b> ) to be acknowledged which may have an array of consequences for the proposed research and its relevance.	[8, 13-16]
Density	Population density has plastic effects on mating behavior and can alter (i.e., increase or decrease) reproductive investment (e.g., gametic transfer, courtship behavior, competition intensity, latency) and oviposition rates. The chosen density for these experiments is not reflective of what may be optimal for industry standards, but rather what is manageable for observation and data collection purposes.	[4, 17]
Sex ratio	The experiment sex ratio proposed is equal number of males to females. Adjusting the ratio to be male-dominant or female-dominant may prove to reduce or intensify oviposition rates; Males experiencing increased or reduced levels of competition instituted by sex ratio may also drive certain reproductive behavioral changes which can affect experiment results.	[4, 17-19]

### **3.4 Future research**

This study is one of the first to examine specific reproductive consequences of senescence in BSF. While this work does start to dig into the variation in reproductive output that occurs in continuous release colony management strategies, it ultimately demonstrates why more work is necessary to better understand how age affects reproductive output to improve BSF breeding programs. One of the paramount ideologies supported by this study is that mating success and eggs produced do not equate to larval output, which is the essential function of maintaining a BSF breeding colony. Additionally, more work is needed to assess legacy effects of adult age in breeding programs. Recent work conducted with *Drosophila melanogaster* (Meigen) (Diptera: Drosophilidae) found that the age of either parent can affect the fitness of the offspring produced [20]. By exploring legacy effects in BSF, there may be a potential to produce reliable trajectories for colony output that can then be utilized for research as well as in industry.

### **3.5 References**

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