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Spotted Wing Drosophila (Drosophila suzukii) in Arkansas: Winter Morphs, Wild Hosts, and Fungal Control

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Spotted Wing Drosophila (*Drosophila suzukii*) in Arkansas:
Winter Morphs, Wild Hosts, and Fungal Control

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Abstract

Drosophila suzukii (Matsumura), or spotted wing drosophila (SWD), is an invasive fruit fly pest that was first found in the United States in 2008. Unlike native *Drosophila,* SWD females have a serrated ovipositor that allows them to attack ripening fruit. Since its introduction, it is unclear how and where they overwinter in Arkansas, what local hosts they utilize and what potential alternative tactics can be used to combat this pest. In states north of Arkansas, winter morphs (WM) of SWD are larger, darker pigmented, and can survive colder temperatures than SWD flies found in the summer. These WM were found in a wide range of alternative fruited hosts during the winter. Many researchers have begun to evaluate efficacy of entomopathogenic fungi against this pest. The objectives of this study were to determine if WM were present in Arkansas; how long into the winter months could SWD be captured and what alternative resources did they utilize in Arkansas; and if *Beauveria bassiana* was effective against this pest. Trap collections from May and October (2015-2017) contained WM female SWD adults that were darker and larger than flies caught in traps from June-August (2015-2017). SWD baited traps were set out from October 2017-May 2018 to see if and how late SWD appeared in Arkansas. Adults were caught until January 2018. Alternative fruit resources they used into the fall and winter months were *Phytolacca americana* L., *Lonicera sempervirens* L., *Rhamnus caroliniana* (Walter) A. Gray, *Lonicera maackii* (Rupr.) Herder and *Callicarpa americana* L. (purple). In the spring, the only alternative host that SWD utilized was *Morus rubra* L. Contact sprays of *B. bassiana* strain GHA at rates from 2.2 x 10^6 to 2.2 x 10^8 spores per ml killed < 2% of SWD flies. This knowledge will help us better understand how SWD is surviving the winter months in Arkansas and the need to conduct future evaluations of other strains of *B. bassiana* on SWD.

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Dedication

To my family that has come before me especially

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Chapter 1: Literature Review on the Biology, Control, and Overwintering of Spotted Wing Drosophila

History

Drosophila suzukii (Matsumura) (Diptera: Drosophilidae), commonly known as spotted wing drosophila (SWD), is an invasive fruit fly pest that was first described in Japan (Hauser 2011, Cini et al. 2012). It was first seen in 1916 attacking cherries but it was not until 1931 was it described by Matsumura (Hauser 2011, Cini et al. 2012). However, many of the phylogenetic relationships have not been resolved causing the true origin of this pest to be unclear (Cini et al. 2012). In the United States, *D. suzukii* was first reported in Hawaii in 1980 but did not raise much of an alarm (Lee et al. 2011b). It wasn't until 2008 that it was detected on the mainland of the United States in Santa Cruz County, California (Walsh 2011). A grower in Santa Cruz County, California noticed fruit fly larvae feeding in his raspberries and sent some off for identification (Hauser 2011). However, initially it was only identified to the genus *Drosophila*, which at that time had no species considered as crop pests in the United States (Hauser 2011). It wasn't until a year later when adult flies were trapped, and specimens were identified as *D. suzukii* which confirmed the presence of SWD in California (Walsh et al. 2011). Since then, it has spread over the United States reaching Arkansas in 2012 and surrounding states (Oklahoma, Kansas and Missouri) the following year (Asplen et al. 2015). It was also reported in Italy and Spain in 2008 and has since spread all over Europe (Asplen et al. 2015). It is now being found in Central and South America (Deprá et al. 2014, Asplen et al. 2015).

Identification/Life Cycle

Although it took time for SWD to be correctly identified here in the United States, it has several distinct characteristics that aid in identification. The generalized characteristics of this pest are a black-striped abdomen, red eyes and is approximately 2-3 mm in length (Calabria et al.

2010, Cini et al. 2012). Sexual dimorphism is apparent in adult SWD. Mature males have a dark spot on each wing tip and two sex combs on their front tibia (Calabria et al. 2010, Cini et al. 2012, Asplen et al. 2015). Young male SWD may be difficult to identify because the characteristic spot on each wing tip is not visible until two days after emergence and resemble other fruit fly species (Cini et al. 2012). Females possess a relatively large serrated ovipositor and lack spots on their wings (Calabria et al. 2010, Cini et al. 2012, Asplen et al. 2015).

The number of days for SWD to complete its life cycle from egg to adult depends on temperature, with lower and upper developmental reproduction thresholds of 10°C and 30°C (Tochen et al. 2014). Degree days (DD) are often used to determine the rate of an insects' development through each growth stage at certain temperatures based on heat accumulation (Murray 2008). The reported thermal constant in cumulative (DD (base 10°C) from oviposition to adult SWD varied from 208 DD (Tochen et al. 2014) to 233 DD (Kanzawa 1939) to 253 DD (Sasaki and Sato 1995). For SWD, as temperatures increase developmental time decreases (Hamby et al. 2016). The optimum temperature between 24 to 26°C allowed SWD to complete its life cycle in 11 days (Kanzawa 1939, Tochen et al. 2014). Females can lay 1-3 eggs in each fruit, depending on fruit maturity (Mitsui et al. 2006, Walsh et al. 2011). Eggs are milky-white and glossy with two breathing filaments that stick out of the fruit (Hauser 2011, Walsh et al. 2011). Larvae develop inside the fruit and are milky-white with black mouthparts and internal organs visible (Walsh et al. 2011). SWD have three instars and can mature in 3-13 days (Kanzawa 1939, Lee et al. 2011b, Walsh et al. 2011, Aspen et al. 2015). The larva pupates inside or outside the fruit and develops to adult from 3-15 days (Walsh et al. 2011). Pupae are an amber color that becomes brown to black when flies are about to emerge (Walsh et al. 2011). There are

7-15 generations of SWD per year depending on climatic conditions (Kanzawa 1939, Walsh et al. 2011).

Hosts/Damage

Flies in the genus *Drosophila have* been very successful in invading new countries (Poyet et al. 2015). Many species within this genus exhibit high fecundity and short generation times and adapt to different climates (Poyet et al. 2015). Since it was first found in the United States, SWD has rapidly spread across the continent and caused great damage to fruit products. The economic losses caused by this pest include: yield loss, increased chemical inputs, increased labor due to monitoring and management, and potential loss of foreign fruit markets (Lee et al. 2011b). In California, Oregon, and Washington combined, SWD strawberry fruit damage could cost $33.4 million per year (Lee et al. 2011b). This is of great concern since most of the United States small fruit production comes from those states (Walsh et al. 2011). It has been suggested that growers could lose more than $500 million annually in strawberries, blueberries, raspberries, blackberries and cherries in those three previously listed states (Walsh et al. 2011). Growers are facing a zero tolerance for infested fruit which leads to additional weekly or more frequent applications of insecticides per year during ripening (Woltz et al. 2014). In Europe, SWD is the 8^{th} species within this genus that has been introduced and has by far been the most damaging (Poyet et al. 2015).

Many *Drosophila* species are considered a nuisance by humans (Asplen et al. 2015). *Drosophila melanogaster* (Meigen) is associated with overripe fruits and can be found in households when fruit is decaying (Asplen et al. 2015). *Drosophila suzukii*, however, attacks intact ripening or ripe fruit (Lee et al. 2011b; Walsh et al. 2011; Asplen et al. 2015). Unlike other fruit flies, SWD females have a serrated ovipositor that allows them to lay eggs in unwounded, ripening and ripe fruit which is earlier than other species (Lee et al. 2011b; Walsh et al. 2011). Firmness and brix levels of fruit affect SWD female's choice of where to lay her eggs. In raspberries, as brix levels increased, more SWD developed (Lee et al. 2011a). Once fruit of blackberry, cherry, strawberry, blueberry, and raspberry start to ripen they were susceptible to SWD (Lee et al. 2011a). Larval feeding creates brown, soft areas visible on the outside of the infested fruits (Walsh et al. 2011). The oviposition wounds and larval feeding potentially provide entry for secondary infections by fungi, bacteria, and other pests (Walsh et al. 2011; Haye et al. 2016).

Drosophila suzukii cause damage to a wide range of hosts including non-crop hosts (Cini et al. 2012, Asplen et al. 2015). Crop hosts include strawberry, raspberry, blackberry, sweet cherry, and mulberry (Cini et al. 2012). When Kanzawa (1939) first observed SWD, he noted that they oviposited most often in cherries, peach, plums, persimmons, strawberries and grapes in June (Kanzawa, 1939). He also noted that they were opportunistic, and adults fed on damaged spoiled fruit that had dropped to the ground (Kanzawa 1939, Walsh et al. 2011). During the winter months in Oregon, SWD flies were observed feeding on overripe/damaged fruits such as persimmons, figs, and apples (Lee et al. 2015). Adults have also been seen feeding on sap from wounded oak trees (Lee et al. 2015).

Alternate hosts (non-crop hosts) can contribute to higher densities and patchy distribution of SWD in nearby crop areas (Lee et al. 2015). Besides keeping SWD population numbers high, alternative hosts can provide a sugar source to sustain SWD into the winter months (Lee et al. 2015). Many growers do not pay adequate attention to weeds and other plants that surround their fields (as opposed to weeds within the field). With SWD, non-crop hosts provide refugia while insecticides are being sprayed on the crop (Lee et al. 2015). Plants within the families of

Rosaceae, Cornaceae, Phytolaccaceae and Elaeagnaceae have been reported as good alternative hosts in Oregon and Michigan (Lee et al. 2015). Woodlands may provide many alternate hosts and overwintering habitat (Pelton et al. 2016). For adults to survive in the winter months, they must find a food source in order to meet energy and metabolic requirements (Kaçar et al. 2016). Growers that have farms surrounded by woodlands report SWD presence earlier than areas that do not have woodlands (Pelton et al. 2016).

Plant host, temperature and relative humidity influence SWD reproductive output (Hamby et al. 2016). SWD population numbers fluctuate throughout the year (Hamby et al. 2016). In early spring, SWD numbers are low but quickly rise as temperatures increase (Hamby et al. 2016). When average daily temperatures rise above 30^{o}C the SWD population levels decline but may resurge once the hottest part of the summer is over (Hamby et al. 2016). In areas where the temperature does not exceed 30^{o}C, fly numbers do not fluctuate as much (Hamby et al. 2016). If suitable habitat become scarce for SWD, they can disperse to find better food sources (Walsh et al. 2011). In Japan, a number of *Drosophila* species, including *D. suzukii*, move from low to high elevations during the summer months to gain access to better resources (Tonia et al. 2016). Moving to higher elevations could also help the flies escape from the heat of the summer (Tonia et al. 2016).

Management

Finding good non-chemical integrated pest management (IPM) strategies against *D. suzukii* is difficult. Growers rely on weekly or more frequent applications of insecticides such as pyrethroids, spinosyns and organophosphates each with differing mode of action (Haye et al. 2016). However, these chemicals must have short pre-harvest intervals because the most effective time for growers to spray is when fruit is ripe (Walsh et al. 2011). Integrating several practices such as cultural and chemical controls can help reduce SWD populations. Ideally, IPM programs help sustain the environment and preserve natural enemies of pest species. Organic growers rely heavily on cultural control for SWD since there is only one insecticide, Entrust (spinosad), approved by the USDA National Organic Standards Board (NOSB) for use against SWD (Racke 2006, Iglesias and Liburd 2016). Once the fruit start to ripen, harvesting every two days and freezing the fruit immediately will help to maintain low levels of SWD infestation in a field (Isaacs et al. 2012, Sial et al. 2018). Sanitation is an important cultural practice to help reduce local density of SWD by removing ripe or overripe fruit from the field (Haye et al. 2016). Once collected, these culled fruits are solarized in plastic bags to kill the larvae and sent to the dump (Haye et al. 2016). Although this is very effective, it is also costly and time consuming (Iglesias and Liburd 2016, Haye et al. 2016).

Identifying alternative hosts for SWD is also important when trying to lessen local density of SWD or manage SWD (Lee et al. 2015). SWD is known to infest non-crop plants such as autumn olive (*Elaeagnus umbellate* Thunb.), mulberry (*Morus rubra* L.), and pokeweed (*Phytolacca americana* L.), all of which can potentially be found around a crop field (Lee et al. 2015). Alternative hosts adjacent a crop can contribute to higher SWD densities (Lee et al. 2015). They allow the flies to develop while crops are not available and infest them when they are ripening and provide a refuge while the crop is being sprayed and then re-infest the field (Lee et al. 2015).

Border sprays can be implemented to help reduce SWD populations around a crop field. This technique can be useful against pests that disperse from surrounding areas, but timing is an important factor to consider. The tactic of spraying field borders may reduce negative impacts on pollinators and natural enemies while still giving some level of protection against SWD (Iglesias and Liburd 2016). Other forms of management include reducing microhabitats that might provide SWD alternative resources (Haye et al. 2016). Pruning understory plants to the ground that are close to the crop field can reduce microhabitats that support SWD reproduction by allowing more sun and airflow, warmer temperatures and lower humidity (Haye et al. 2016).

Some growers are starting to grow their crops in high tunnels that allow them to manipulate harvest date and reduce potential diseases (Asplen et al. 2015). The high tunnels use insect netting to exclude SWD (Asplen et al. 2015, Herrera 2017). All season, Herrera (2017) excluded SWD and prevented SWD larvae in blackberry fruit in a high tunnel (60 m x 7.3 m x 3.6 m), with roof covered by greenhouse grade poly film and 1.5 m lower sides and ends covered with insect exclusion netting (80 gm, 1 mm x 0.06 mm mesh) (Tek-Knit Industries, Quebec Canada). Along with high tunnels, growers are also advised to use sanitation measures to help reduced possible breeding sites for SWD (Walsh et al. 2011). Combining methods can also help reduce SWD infestations. Leach et al. (2016) found that over a two-year period, SWD larvae in fruit were reduced when exclusion netting was combined with insecticides (Leach et al. 2016). With high tunnel exclusion netting, there was an 82% drop in eggs, 74% reduction in larvae and 65% drop in adults (Leach et al. 2016, Herrera 2017). This exclusion netting approach delays the infestation of SWD and can reduce the number of pesticide applications, reduce potential resistance and lowers the risk of harming pollinators (Leach et al. 2016).

Biological control might play a role in managing SWD (Haye et al. 2016). Native predators such as *Orius* (Hemiptera: Anthocoridae) have been seen to feed on SWD larvae but probably do not have much potential in suppressing the large SWD population (Walsh et al. 2011; Asplen et al. 2015). There are multiple parasitoids that attack various stages of other *Drosophila* species. Larval parasitoids, such as *Leptopilina heterotoma* (Thompson), *L. boulardi*

Barbotin (Hymenoptera: Figitidae), and *Asobara tabida* (Nees) (Hymenoptera: Braconidae), attack other *Drosophila* species, but are unable to attack SWD larvae since they have an immune response to parasitoids (Haye et al. 2016). In the United States, the parasitism rate of SWD was low by the native pupal parasitoid *Pachycrepoideus vindemmiae* (Rondani) (Hymenoptera: Pteromalidae) and larval parasitoid (*Asobara* sp.) (Girod et al. 2018). Pupal parasitoids, *Trichopria drosophilae* Perkins (Hymenoptera: Diapriidae) and *P. vindemmiae*, have been successful against SWD under lab conditions (Haye et al. 2016). Augmentative releases of *T. drosophilae* before fruit ripening were successful in a greenhouse (Haye et al. 2016). Girod et al. (2018) noted that *Ganaspis* sp. collected from China and Japan infested and developed successfully in SWD larvae. Under laboratory conditions, *Ganaspis* sp. parasitized more SWD larvae inside fresh blueberries than larvae in artificial diet suggesting that they may have a specialization for SWD (Girod et al. 2018). *Leptopilina japonica* Novkovic and Kimura (Hymenoptera: Figitidae) and *Asobara japonica* Belokobylskij (Hymenoptera: Braconidae) attack a wide range of *Drosophila* larvae in ripe and rotten fruits, mushrooms and decayed leaves allowing them to find SWD on multiple substrates (such as artificial diet in the lab) (Girod et al. 2018). Girod et al. (2018) stated, "Classical biological control, introducing Asian parasitoid wasps specialized in *D. suzukii*, may provide a sustainable and area-wide long-term solution."

Beauveria bassiana

Since the invasion of SWD, growers have had to add 5-8 weekly applications of insecticides per season due to a zero tolerance for infested fruit (Van Timmeren and Isaacs 2013, Woltz et al. 2014). Due to this, researchers have started to investigate alternatives to pesticides. Alternatives include entomopathogenic fungi, entomopathogenic nematodes and potential predators. Two fungi, *B. bassiana* (Balsamo) Vuillemin and *Isaria fumosorosea* Wize were known to affect adult *Aedes aegypti* (L.) (Diptera: Culicidae), house flies, *Musca domestica* L. (Diptera: Muscidae), and tropical and temperate species of fruit flies (Diptera: Tephritidae) (Cossentine et al. 2016). Both *B. bassiana* and *I. fumosorosea* have a dose dependent impact on SWD mortality (Cossentine et al. 2016). *Beauveria bassiana* is a well-studied entomopathogenic fungi that can infect more than 700 different species (Li et al. 2011). It causes "white muscardine" disease of silkworms which once threatened silk production (Webster and Weber 2007). *Beauveria bassiana* has been used to help control agricultural and forest pests and infects larvae most insects (Li et al. 2011). *Beauveria bassiana* is distributed in the soil and can infect species of Coleoptera, Diptera, and Lepidoptera (Webster and Weber 2007). When an individual insect has been infected by *B. bassiana*, mycelium grows out of the hosts and covers the body in hyphae bearing conidia creating what looks like a white dust (Webster and Weber 2007). There are several *B. bassiana* formulations on the market using different strains that are available for growers to utilize. Having another alternative control method for growers to use would be beneficial in combating this pest and help in the fight against SWD resistance to pesticides.

Results have differed in how effective *B. bassiana* is for control of *D. suzukii*. It does cause population decreases but has not reduced a SWD population below economically damaging level (Gargani et al. 2013; Cuthbertson et al. 2014). Since SWD have a short development time, the next generation of larvae are often found the following week after a fungal spray (Cuthbertson et al. 2014). Conidia of *B. bassiana* were found to attach to the pretarsi of SWD after 48 hours post exposure to treated paper (Cossentine et al. 2016). This exposure killed 44% of SWD by 7 days after exposure to *B. bassiana* (Cuthbertson et al. 2014). Research by Woltz et al. (2014) found that *B. bassiana* had no significant effect on fly mortality. Different entomopathogenic fungal species isolates can vary in virulence to a given hosts (Woltz et al. 2014) which may help explain the differing results. In *D. melanogaster,* the impact of *B. bassiana* effectiveness was reduced when the flies were infected with *Wolbachia* bacteria (Panteleeve et al. 2007)*. Wolbachia*, is a symbiotic cytoplasmic bacteria that can cause reproductive alterations in arthropods (Werren et al. 1995; Mazzetto et al. 2015). A number of effects caused by *Wolbachia* have been observed in *Drosophila* species (Mazzetto et al. 2015). A *Wolbachia* endosymbiont sequenced from SWD positively affected female fecundity (Mazzetto et al. 2015). In Oregon, 20% of the SWD collected were infected with *Wolbachia* (Tochen et al. 2014)*.* Since *D. suzukii* belongs in the subgroup within the *D. melanogaster* species group (Asplen et al. 2015), it is possible that *Wolbachia* could also reduce how effective *B. bassiana* is on *D. suzukii.*

Winter Morphs/Cold tolerance

Spotted wing drosophila inhabits a wide climatic range from subtropical to continental regions (Shearer et al. 2016). In Japan, where SWD were first described, the lower temperatures killing 50% (LT_{50}) of females and males were -1.6°C and -0.3°C, respectively (Kimura, 2004). When it was first discovered in the United States, it was predicted that SWD would not be able to survive in colder climates (Stephens et al. 2015). However, SWD is an established pest in all the northern states and Canada (Shearer et al. 2016). For SWD to become a well-established pest in these colder areas, they must have ways to survive the cold winter temperatures. Insects have several strategies that allow them to be cold tolerant (Shearer et al. 2016), such as rapid cold-hardening which takes minutes or hours, or seasonal cold-hardening that takes days or weeks (Wallingford et al. 2016). Wallingford and Loeb (2016) defines these terms *"rapid cold-hardening refers to pre-exposure in the short-term (hours) while gradual acclimation refers to pre-exposure in the long term (days or weeks), while developmental acclimation refers to* *gradual acclimation experienced during ontogeny."* Seasonal cold-hardening can be important for insects that need to enter winter diapause (Wallingford et al. 2016). Since SWD can survive below-zero temperatures, it suggests that they rely on a freeze avoidance mechanism which maintains body fluids as liquids and keeps them from freezing (Sinclair et al. 2003; Wallingford et al. 2016). Insects can also have multiple phenotypes as a response to a change in the environmental conditions known as phenotypic plasticity (Moczek 2010, Shearer et al. 2016). It has been a long-standing taxonomic problem because as environmental conditions change, a single genotype can respond by having multiple phenotypes (David et at. 1990). Adult insect diapause is usually associated with a delay in reproduction (Nylin 2013). Reproductive diapause allows an individual to temporarily cease reproduction to preserve energy and allocate resources to survive unfavorable conditions (Shearer et al. 2016). Local adaptations allow insects to facilitate their survival and reproduction across a wide range of local environmental conditions. Seasonal cues and hormones can aid in the regulation of diapause and seasonal morphs of insects (Nylin 2013).

All stages of SWD experience winter mortality with temperatures below freezing, but not total eradication of the population (Walsh et al. 2011). However, previous research has suggested that summer-morph (SM) adults would not be able to survive in cold temperatures, but that winter-morphs (WM) might be able to (Stephens et al. 2015). In other *Drosophila* spp., including *D. melanogaster*, dark WM are known to aid in the overwintering of the insect (Stephens et al. 2015). Chill-susceptibility appears to be an ancestral strategy for cold tolerance in the *melanogaster* subgroup of *Drosophila*, which is the group to where *D. suzukii* belongs (Jakobs et al. 2016). Developmental conditions can affect how the fly develops (Wallingford and Loeb 2016). Exposure of *D. melanogaster* to lower temperatures resulted in higher black pigmentation of the last three tergites of the fly's abdomen (David et al. 1990). When larvae were developed under winter conditions (i.e. short days and low temperatures), the adults that emerged displayed WM characteristics (Toxopeus et al. 2016). They also found cuticular darkening when larvae were exposed to both long and short days at 11°C. This did not occur when larvae were exposed to the two different day lengths at 15°C and 21.5°C, suggesting that temperature is the cue for increased melanization rather than photoperiod (Toxopeus et al. 2016). Many insects follow a 'temperature-size rule': as temperatures increase, body size decreases and development rates increase (Jakobs et al. 2016). When comparing summer and winter conditions, SWD reared under summer conditions were smaller and had less pigmentation than flies reared under winter conditions (Wallingford and Loeb 2016). Female SWD caught in October and November had higher pigmentation than those caught in August and September suggesting a winter state (Wallingford and Loeb 2016). As the average temperatures decreased, SWD caught in the field also had longer wing lengths (Shearer et al. 2016). Lab reared SM were smaller than WM when looking at thorax length, wing length and the ratio between the two (Wallingford and Loeb 2016).

Winter-acclimated flies were more cold tolerant than summer-acclimated flies suggesting that they could survive colder temperatures (Stephen et al. 2015, Toxopeus et al. 2016). Un-acclimated flies had high mortality rates when exposed to suboptimal temperatures (Winman et al. 2016). As temperatures decreased below 10°C, adult SWD lifespan also progressively decreased (Dalton et al. 2011). All SWD larvae and eggs cannot survive the winter temperatures (0°C or colder) (Kaçar et al. 2016, Jakobs et al. 2017). Although larvae that developed under winter conditions emerged as adults with winter characteristics, the larvae cannot survive cold climates even if they are acclimated (Jakobs et al. 2017). Rossi-Stacconi et al. (2016) found

SWD adults fly throughout the winter in Trentino (Italy), even when the average minimum temperature was below 0°C. Other *Drosophila* species were reported to overwinter in man-made structures or under leaf litter (Wallingford and Loeb 2016). Although during the winter months, SWD flies may not be readily trapped, individuals can quickly become active when conditions are more favorable to find food (Rossi-Stacconi et al. 2016).

Diapause

Along with overwintering and winter morphs, it is often speculated that adult SWD may go into diapause to survive the winter months. Diapause helps insects survive seasonal fluctuating resources, diversify in tropical habitats, and colonize temperate and polar regions (Koštál 2006). Diapause can be found in many *Drosophila* species including *D. melanogaster* which appears to have a photoperiod regulated ovarian diapause (Saunders et al. 1989; Kubrak et al. 2014; Shearer et al. 2016). A change in physiology, gene expression involving metabolism in *D. melanogaster* help them enter a state of dormancy (Kubrak et al. 2014). Winter-acclimated SWD were found to have higher stress tolerance and suppression of ovarian development. However, the researchers of this study did not believe that this represented true diapause since it is seemed to be only regulated by temperature (Toxopeus et al. 2016). Since SWD can survive below-zero temperatures, it suggests that they rely on a freeze avoidance mechanism which maintains body fluids as liquids and keeps them from freezing (Sinclair et al. 2003; Wallingford et al. 2016). In China, during the months of April to June an increase in ovary maturation was noticed in female SWD while from July to November it decreased. Adult females that were caught during winter had immature ovaries suggesting that temperature and photoperiod played an important role in their development (Zhai et al. 2016). Wallingford et al. (2016) found that under lab conditions, low temperatures had the strongest effect on egg maturation, but photoperiod is an important stimulus for inducing diapause in SWD.

During the first colder months of the year, males are not often caught which suggests that females mate before the cooler temperatures arrive in the fall and lay those eggs the following spring (Dalton et al. 2011, Rossi-Stacconi et al. 2016). Females have been shown to be more cold tolerant than males (Plantamp et al. 2016). Throughout the year, the ratio between males and females fluctuates (Rossi-Stacconi et al. 2016). The sex ratio of SWD adults captured is skewed to females during the first cold months of the year (Rossi-Stacconi et al. 2016). As temperatures start to warm more males are present, but during the hot summer months the ratio shifts to more females (Rossi-Stacconi et al. 2016). During the fall months, the ratio switches once again and favors males until nearly the end of the season (Rossi-Stacconi et al. 2016).

Overwintering sites

Although SWD can develop into WM, farms in areas that had wooded areas nearby could potentially provide resources for SWD into the fall (Pelton et al. 2016). They found that farms surrounded with high amounts of woodland caught flies in traps one week earlier in the spring than farms surrounded by low levels of woodland. Alternative hosts are not harvested like cultivated fruit and persist longer into the fall providing SWD with resources to reproduce and feed on in the winter (Pelton et al. 2016). Pelton et al. (2016) had study sites in Wisconsin, Minnesota and Michigan where SWD were trapped in woody areas into the first part of November suggesting that these woody areas provided resources for SWD. Kaçar et al. (2016) found that SWD could feed on apple juice or cherry juice and that food was critical for adult survival. In some areas of California and North Carolina, SWD can be trapped year-round

(Pelton et al. 2016). Humidity, oviposition substrate, food resources, and temperature all affect the life span of SWD (Hamby et al. 2016).

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Chapter 2: Winter Morphs of Spotted Wing Drosophila in Arkansas

Abstract

Drosophila suzukii (Matsumura) or spotted wing drosophila (SWD) is an invasive fruit fly pest that has become well-established in the United States. Female SWD have a serrated ovipositor that allows them to oviposit eggs in ripening or ripe fruit. Since its introduction into the United States, it has caused millions of dollars in crop loss. Winter temperatures in the northwest region of the United States were thought to prevent SWD from establishing, however, populations have persisted year around. Flies that develop under cooler winter conditions have increased pigmentation. There is a winter morph (WM) of SWD which appears to be larger with darker pigmentation compared to its summer morph and is thought to help SWD overwinter. Winter morphs have been found in Minnesota and Oregon, but little is known where else they can occur. A study was conducted in Arkansas and Tennessee to determine the presence of WM in these more southern areas. Three locations in Arkansas (2015-2017) and one location in Tennessee (2013) were monitored for WM from the spring through fall. The wing and thorax length of female SWD from each sample were measured as well as the ratio between the two. A pigmentation score was created for abdominal tergites 3, 4, and 5 ranging from 0 (no pigmentation) to 10 (full pigmentation). In Arkansas, SWD in October and May had higher pigmentation scores and larger wings than in any other months. In Tennessee, SWD in December had the highest pigmentation and wing lengths compared to any other months. Temperature data was collected for the 2017 sample year to see when temperatures dropped below the optimal range (20-25°C) for SWD to be active. The temperatures in October began to drop below the optimal range for SWD to be active and increased pigmentation was observed.

Keywords: spotted wing drosophila, winter morphs, overwintering

Introduction

Drosophila suzukii (Matsumura) or spotted wing drosophila (SWD) was first identified in the United States in 2008 (Hauser 2011). Unlike native *Drosophila* species, female SWD have a serrated ovipositor that allows egg laying in ripe fruit (Hauser 2011, Walsh et al. 2011, Cini et al. 2012). Since the introduction of SWD, it was estimated that growers could lose more than $33 million in strawberries and $56 million in blueberries per year (Lee et al. 2011). Spotted wing drosophila can also utilize a wide array of non-crop hosts including mulberry (*Morus* spp.), pokeweed (*Phytolacca americana* L.) and honeysuckle (*Lonicera* spp.) (Lee et al. 2015). These alternative hosts are commonly found in or adjacent to susceptible crop fields and can provide additional resources and food into the winter months (Lee et al. 2015).

When SWD was first found on the west coast of the United States, it was thought that they would not be able to survive extended periods of cold temperatures (below 10°C) in the winter which would prevent them from establishing (Dalton et al. 2011, Shearer et al. 2016). Despite these predictions, the fly has become well adapted to cold conditions in areas such as Washington, Michigan, Canada and Italy (Shearer et al. 2016, Tonina et al. 2016). The optimal range for SWD to be active is between 20-25°C but can reproduce between 10-32°C (Kanzawa 1939, Cini et al. 2012). However, it is still unclear where and how SWD overwinter in these colder areas.

Insects that overwinter must be able to survive long periods of cold or milder conditions. Overwintering insects often go into what is known as diapause (Toxopeus et al. 2016). Diapause is when normal development is suppressed by mediated mechanisms usually during unfavorable conditions (Košťál 2004). Adult diapause (or reproductive diapause) is associated with delayed reproduction (Nylin 2013). Female insects can go into reproductive diapause which stops the process of oogenesis and vitellogenesis (Baker and Russel 2009). Many species of *Drosophila,* such as *D. melanogaster* (Meigen), have been observed to undergo diapause (Saunders et al. 1989, Shearer et al. 2016). Given that *D. melanogaster* is a close relative, it is suspected that SWD may exhibit the same behavior (Shearer et al. 2016). In Hokkaido, Japan, high rates of reproductively immature adult SWD females were caught in the months leading up to winter, suggesting that diapause may occur (Mitsui et al. 2010, Shearer et al. 2016). In Oregon, females caught in October-December had fewer eggs than those in August and September. Diapause allows for the conservation of resources, such as producing fewer eggs, until favorable conditions (i.e. warmer temperatures) are met (Shearer et al. 2016, Wallingford et al. 2016).

Insects have been known to adapt physiologically when faced with different environmental conditions. Development temperatures, diet and genotype can all play a role in body size and pigmentation of an insect (Wallingford and Loeb 2016). For example, pigmentation has been known to increase when insects are exposed to colder temperatures (David et al. 1990). Many insects also follow a 'temperature-size rule', meaning that as temperatures increase, the rate of development increases and body size decreases (Kingsolver and Huey 2008; Jakobs et al. 2017). Some *Drosophila* species have been observed to adapt through multiple phenotypes that have different bristle numbers and wing and thoracic lengths. Variation in pigmentation due to temperature has been known to occur in female *D. melanogaster* (David et al. 1990).

In SWD, developmentally acclimated winter morphs (WM) are larger and darker than summer morphs (SM) (Wallingford and Loeb 2016). In Minnesota and Oregon, SWD WM have been caught in mid-October (Stephens et al. 2015; Hamby et al. 2016). It has been suggested that winter morphs play a role in how adult SWD can become active in the winter months (Rossi-

Stacconi et al. 2016). However, one study found that WM and SM could not survive cold temperatures unless they found a way to escape the cold (Stephens et al. 2015). In a lab study, SWD eggs and larvae reared under autumn or winter conditions (i.e. low temperatures and short photoperiods) produced WM adults (Stephens et al. 2015). Since they were reared under autumn or winter conditions these adults were believed to be cold acclimated and became more cold tolerant than SM (Stephens et al. 2015). Acclimated adult SWD survived up to 88 days at 10°C with mortality rising as temperatures dropped below 10°C (Dalton et al. 2011). With acclimated adults being able to survive colder temperatures, it would take even colder temperatures and a longer time frame to kill them off during the winter. In the Pacific northwest, it would take 56 chilling degree-days with temperatures at or below 11.6°C to reach 20% mortality of adult SWD (Coop 2011). Degree days are often used to predict development patterns based on heat accumulation (Murray 2008). For SWD as temperatures decrease, developmental time increases (Hamby et al. 2016). Insects require a certain amount of heat accumulation to reach a certain life stage which allows growers to predict the best time to treat for a pest (Murray 2008). Lower development temperatures slow the growth of the larvae allowing for larger imaginal tissues which means larger adults emerge (Wallingford and Loeb 2016). Pigmentation, wing length, thorax length and a ratio of wing: thorax have been looked at to evaluate how different conditions affect the morphology of the adult fly (Wallingford and Loeb 2016). Female SWD caught in October and November had higher pigmentation than females caught in August and September (Wallingford and Loeb 2016). Along with pigmentation, female SWD caught in October and November had larger wing length and wing: thorax ratios than those caught in August and September (Wallingford and Loeb 2016). In this study, female SWD were caught through the growing season and into the fall to see if WMs were present in Arkansas and

Tennessee. Daily temperatures were recorded for the 2017 samples to see when temperatures started to drop in the fall.

Materials and Methods

Locations

Three locations were used from Arkansas:1) the Arkansas Agricultural Research and Extension Center farm (AAREC, Fayetteville, 2015-2016) (36°05'N, 94°10'W), 2) the University of Arkansas System Division of Agriculture Fruit Research Station (UAFRS, 2015-2016) (35°32'N, 93°24'W), and 3) Private farm near Tontitown (Farm 1, 2017). Samples from 2013 were received from one site located at the Soil, Plant, and Pest Center in Nashville, Tennessee (36°03'N, 86°44'W). Collection periods varied among location and year **(Table 1)**.

The SWD flies at each Arkansas location were caught using a one-liter clear plastic deli cup with about 20 (5 mm diameter) holes on the upper half of the container two thirds the way around. Red and black duct tape strips were added to the trap to increase visual attractiveness to SWD (Lee et al. 2012). One Scentry SWD pouch lure was attached to the underside of the lid. Apple cider vinegar was used as a drowning solution. The SWD flies at the Tennessee location were caught using clear plastic deli cups containing 150 ml water, 5 g sugar (Domino Sugars, Iselin, NJ) and 10 g yeast (Red Star Brand dry active yeast). Forty SWD females in 2015-2016 and 20 SWD females in 2017 were observed each month per location for the presence of the SWD winter morph. In 2013, only 12 SWD females were looked at for WMs.

Measurements

The SWD females were collected monthly between the months of May and December and each was photographed with a DinoCapture 2.0 digital microscope (AnMo Electronics Corporation, New Taipei City, Taiwan). Wing and thorax measurements were recorded using the

DinoCapture 2.0 software (AnMo Electronics Corporation, New Taipei City, Taiwan) **(Fig. 1A-B)**. Wing and thorax lengths were determined using methods developed by Wallingford and Loeb (2016). The distance between the anterior portion of the wing base and the distal wing margin at the L3 vein was recorded for wing length **(Fig. 2A).** The distance between the anterior margin of the thorax (propleuron) to the posterior tip where the thorax attaches to the abdomen was recorded for thorax length **(Fig. 2B)**. The pigmentation of each female SWD was scored and recorded based on a similar scoring technique developed for *D. melanogaster* by David et al. (1990). The general trend of pigmentation was better observed when the scores of the last three tergites were scored (David et al. 1990). This technique was modified for SWD by Wallingford and Loeb (2016). A modification of their technique was used for this study. With many fly specimens, the last (sixth) tergite was hidden and scoring could not be done. It was decided to not use the last three tergites, but to use tergites three, four, and five **(Fig. 3)**. The pigmentation of three abdominal tergites of SWD were scored ranging from 0 (no pigmentation color) to 10 (complete pigmentation color) **(Fig. 4).** The scores were then summed to get a total out of 30. In addition, the 2017 maximum daily temperatures were derived from the online phenology and degree-day model site (uspest.org 2017) that accesses daily maximum and minimum temperatures from weather loggers near the Farm 1 in Tontitown and UAFRS.

Statistical analysis

Statistics were done similarly to that of Wallingford and Leob (2016) study. Differences among sample dates in the mean measurements of wing, thorax, wing: thorax ratio and pigmentation scores were done with one-way ANOVA and mean separation by Tukey-Kramer HSD.

The SWD fly measurement data from the UAFRS (2015-2016) was the only location that was normally distributed. Log transformation was done on the data and it was still not normally distributed, so several outliers were removed to normalize the data. The SWD wing measurement data from the AAREC in Fayetteville (2015-2016) were not normally distributed (p<0.02), but since the p-value was close to being non-significant, it was decided not to throw any outlier data points out. Wing and thorax measurements of SWD flies from the UAFRS (2017) were not normally distributed (p<0.04 and p<0.02, respectively). Again, since they were close to being not significant, it was decided that the outliers would not be taken out of the data set.

Results

Pigmentation

In Nashville, Tennessee, SWD in December had higher pigmentation than any other month between August-November ($F_{3,39}$=27.8; P<0.0001) **(Fig. 5, Table 2)**. In 2015-2016 at the AAREC-farm, July SWD fly samples had significantly lower pigmentation score than September and October ($F_{3,94}$=7.8; P<0.0001) **(Fig. 6, Table 2)**. Due to the poor preservation of the flies, pigmentation scores could not be determined for UAFRS 2015-2016. In 2017 at UAFRS, there was no difference in SWD fly pigmentation between the months ($F_{3,76}$=1.4; P<0.26) **(Fig. 7, Table 2)**. However, at Farm 1 in Tontitown, SWD flies caught in May and October had significantly higher pigmentation scores (<11) than June, July and August (>5) ($F_{4,83}$=13.2; df=4, 83; P<0.0001) **(Fig. 8, Table 2)**.

Wing and Thorax

Wing lengths of flies caught in December were significantly longer than any other months in Nashville, Tennessee ($F_{3,39}$=15.9; P <0.0001). December and September were the only months significantly different from each other in thorax length ($F_{3,39}$=3.3; P<0.03). The ratio between wing: thorax had no significant differences between any of the months in Nashville, Tennessee ($F_{3,39}$=2.5; P>0.07) **(Table 2)**. In Arkansas, females caught in October (2015) and June (2016) at AAREC-farm had larger wing ($F_{3,94}$=11.6; P<0.0001) and thorax lengths ($F_{3,94}$=10.0; P<0.0001) than in other months. There was no difference found between wing: thorax ratio between any of the months ($F_{3,94}$=1.1; P>0.33) **(Table 2)**. For UAFRS in 2015-2016, July and August had significantly smaller wings ($F_{5,197}$=29.8; P<0.0001) and thorax measurements ($F_{5,197}$=13.2; P<0.0001) than the other months and May and October had the longest. October and June were the only two months different from each other in wing: thorax ratio ($F_{5,197}$=3.0; P<0.01) **(Table 2)**. In 2017 at UAFRS, flies caught in May and June had the smallest wing ($F_{3,76}$=22.7; P<0.0001) and thorax ($F_{3,76}$=20.4; P<0.0001) measurements compared to July and August. There was also no difference between the months and wing: thorax ratio ($F_{3,76}$=1.2; P>0.31) **(Table 2)**. At Farm 1 in Tontitown, SWD flies caught in October 2017 had the longest wing ($F_{4,83}$=15.6; P<0.0001) and thorax ($F_{4,83}$=10.9; P<0.0001) lengths compared to other months. However, there was no difference found between wing: thorax ratio between the months ($F_{4,83}$=1.2; P>0.33) **(Table 2)**.

Discussion

Nashville, Tennessee was the only location in my study that had SWD female flies caught in December. In August at Nashville, mean pigmentation score was two. By December, the mean increased to 25 **(Table 2; Fig. 5)**. At AAREC (2015) and Farm 1 (2017) locations in Arkansas, SWD caught in October had increased pigmentation (<7) and longer wing lengths (<2.2mm). These results are similar to the study conducted by Wallingford and Loeb (2016) that found female SWD caught in New York during October and November had larger wings and darker pigmentation than those caught in August and September. However, female SWD caught in October and May, showed no differences in wing, thorax length and pigmentation **(Table 2; Fig. 6, 8)**. The measurements of SWD flies were not significantly different between May and fall collections. Females caught in the fall months were most likely the ones overwintering and the first to emerge in the spring since it is believed that adult females are the ones overwintering (Kanzawa, 1939; Walsh et al. 2011).

One unexpected finding was that SWD wing length in July 2017 was not significantly smaller than the SWD wing length in October at Farm 1**(Table 2)**. Wing length at UAFRS was longer in July than any other months **(Table 2)**. These increases in wing length may be due to an increase in resources available in July in Arkansas. July is prime time for many Arkansas fruit crops, such as blackberries, to become ripe along with non-crop hosts. Since resources are not limiting, the flies may be able to allocate resources to different areas of their bodies. It is believed that larger wing size can increase dispersion capabilities (Shearer et. al 2016). Spotted wing drosophila can complete multiple generations during a crop season allowing populations to increase quickly and overlap in generations (Cini et al. 2012). When SWD populations are very high adults may need to find new resources. With a greater wing span they may be better able to find new resources.

In 2017, SWD from UAFRS were found to have no significant differences in pigmentation between May and the summer months (June-July), whereas SWD at the Farm 1 near Tontitown, AR did **(Table 2; Fig. 7-8)**. UAFRS is located about 94 miles southeast of Fayetteville and has a river effect that can influence temperatures and humidity in that area. When looking at maximum daily temperatures of these two areas, UAFRS had warmer temperatures **(Fig. 9)**. It has been suggested that temperature is a cue for increased melanization and not photoperiod (Toxopeus et al. 2016). In May, UAFRS was on average 4°C warmer than

Farm 1. With May being warmer, it might explain why there was no difference in pigmentation scores between May and the summer months at this location. SWD development is temperature dependent and as temperature increases, development time decreases (Hamby et al. 2016). With temperatures being warmer at UAFRS earlier in 2017, SWD were not as exposed to cold development temperatures and can develop faster and have less need to be cold tolerant.

Although differences in pigmentation and wing length were found in both Tennessee and Arkansas, more studies need to be done to understand WMs in these areas. Wing length varied throughout the year with it increasing toward the fall months. However, neither the wing or thorax length was different among the SWD flies caught in July or October. These results suggest that size alone may not be a good indicator of a WM. Winter morphs have been generally characterized by having increased pigmentation and a larger body size, however there are currently not any defining characteristics of SWD winter morphs. Based on what is known about WM, in this study the SWD females caught between October to December had the characteristic, darker pigmentation, of WMs at all locations. Temperatures started to drop in October of 2017 and at Farm 1 an increase in pigmentation was observed **(Table 2; Fig. 9)**. Winter morphs in other parts of the United States have been caught in mid-October (Stephens et al. 2015; Hamby et al. 2016) which supports the findings in Arkansas and Tennessee.

To improve the identification of WM, more SWD should be sampled during the winter months to compare all potential morphological differences and provide a set of characteristics. Understanding that WM are present in an area gives us a better knowledge of how they are surviving the colder temperatures during the winter. Wallingford and Loeb (2016) noted that a change in developmental conditions affect adult morphology and can also play a role in reproductive diapause. Moving forward, it will be important to study WMs that are trapped to see if they show the signs of reproductive diapause. Diapause is induced by cooler temperatures and shorter photoperiods which also plays a role in SWD developing into a WM (Koštál 2006; Wallingford and Loeb 2016). Winter morphs may be a physical appearance that indicates a SWD preparing for diapause. More still needs to be studied to better understand how WMs and diapause may aid in the overwintering of SWD.

If WMs are found to be present, we can start to develop a chilling degree day model to predict the percentage of adult mortality through the winter. In the Pacific Northwest, it was predicted that it takes 109 days at or below 11.6°C to get 50% mortality of adult flies during the winter (Coop, 2011). Developing a spring emergence model will allow growers to get an idea about how soon they might see SWD entering their fields in the spring.

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Appendix

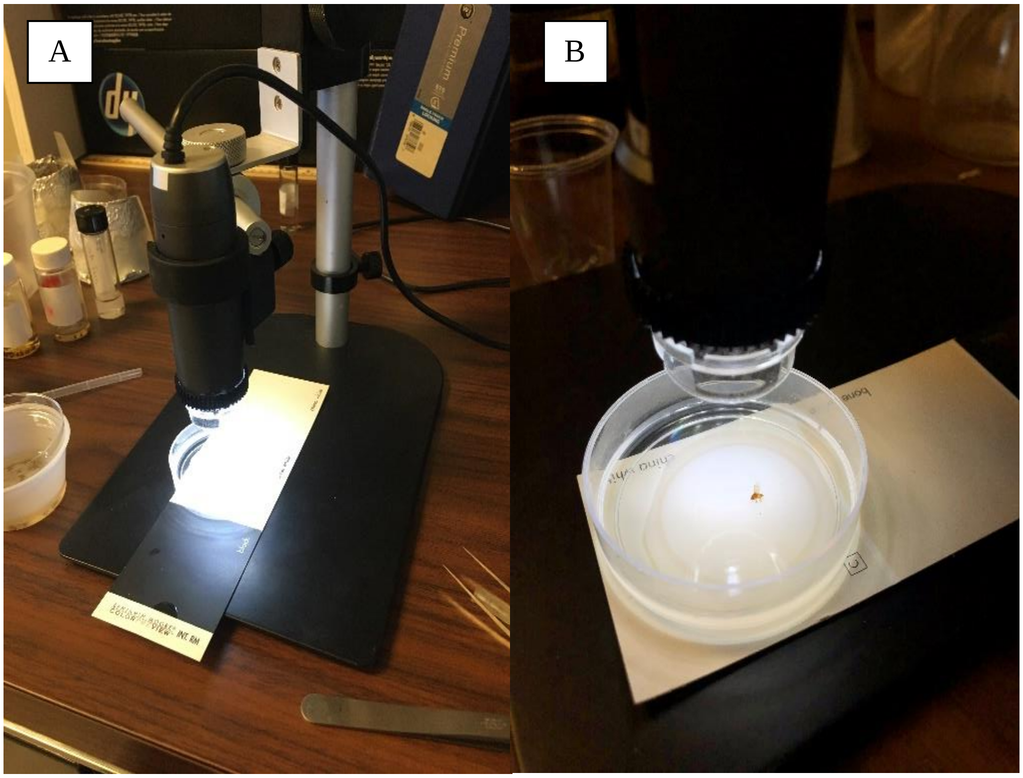

Figure 1: DinoCapture 2.0 used to take measurments and photographs of spotted wing drosophila (SWD) A) set up of the microscope and B) close up of camera and SWD specimen.

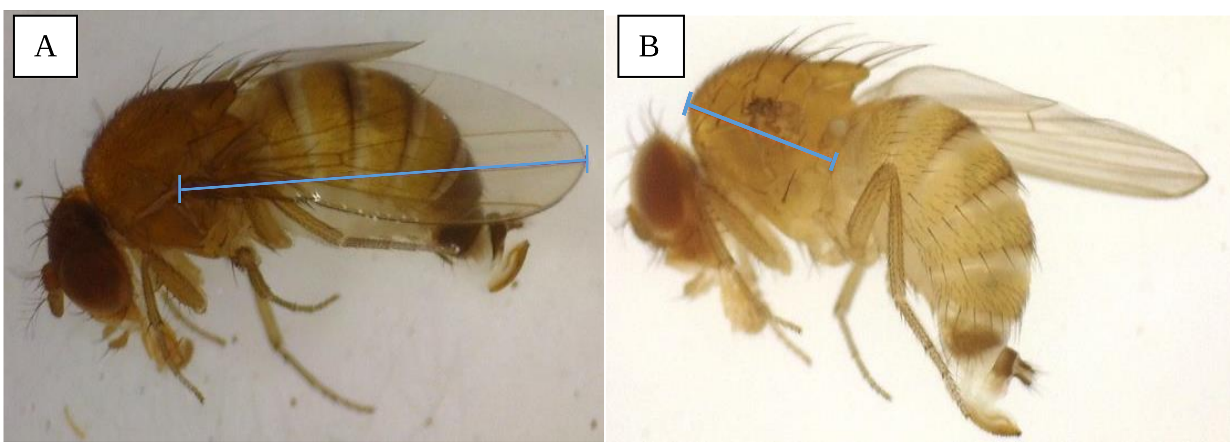

Figure 2: Female spotted wing drosophila A) wing measurement from where it attaches to the thorax to the L3 vein and B) thorax measurement from the anterior margin of the thorax (propleuron) to the posterior tip of the scutellum.

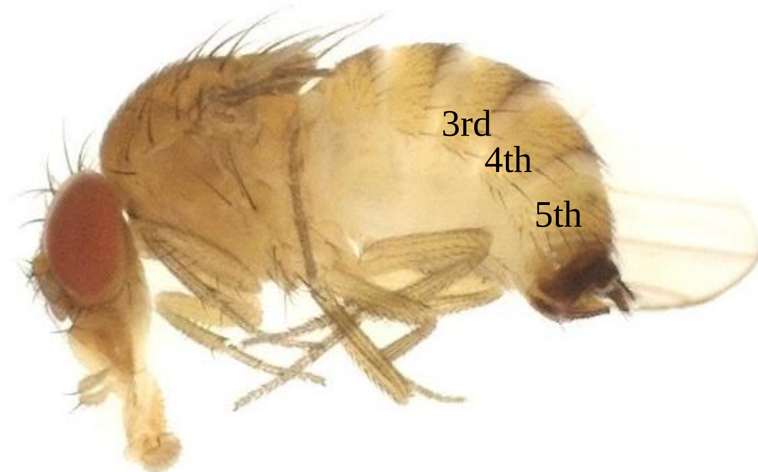

Figure 3: Tergites 3, 4, and 5 of female spotted wing drosophila abdomen that were used in scoring pigmentation.

Figure 4: Example of a female spotted wing drosophila fly with pigmentation scores noted on the tergites 3, 4, and 5 of the abdomen (total score is 16).

Table 1: Collection periods for each location by year.

Year	Location	Collection period
2013	Nashville, Tennessee	Aug. 9-20
		Sept. 6-27
		Oct./Nov.
		Dec. 9-19
2015-2016	AAREC-Fayetteville, AR	Sept. 17, 2015
		Oct. 1-22, 2015
		June 3-24, 2016
		July 29, 2016
	UAFRS-Clarksville, AR	Sept. 9-23, 2015
		Oct. 8, 2015
		May 25, 2016
		June 17-22, 2016
		July 29, 2016
		Aug. 5-25, 2016
2017	UAFRS-Clarksville, AR	May 13-23
		June 9-23
		July 6-20
		Aug. 4-18
	Farm 1-Tontitown, AR	May 23
		June 1-29
		July 13-27
		Aug. 10-24
		Oct. 4

Table 2: Mean (± SE) pigmentation score (0-30), wing and thorax length, and wing to thorax ratio of adult female spotted wing drosophila at different locations by year and month.

Year	Location	Collection period	Pigmentation score	Wing (mm)	Thorax (mm)	Wing: Thorax (mm)
2013	Tennessee	Aug.	2.0 ± 2.1 c	2.2 ± 0.08 b	0.9 ± 0.03 ab	2.7 ± 0.12 a
		Sept.	8.4 ± 1.7 bc	2.0 ± 0.06 b	0.8 ± 0.03 b	2.6 ± 0.10 a
		Oct./Nov.	12.0 ± 1.8 b	2.2 ± 0.07 b	0.8 ± 0.03 ab	2.7 ± 0.10 a
		Dec.	25.0 ± 1.7 a	2.6 ± 0.06 a	0.9 ± 0.03 a	3.0 ± 0.10 a
2015-2016	AAREC	Sept. 2015	8.0 ± 1.6 a	2.1 ± 0.06 b	0.8 ± 0.03 b	2.6 ± 0.06 a
		Oct. 2015	7.0 ± 1.1 a	2.3 ± 0.04 a	0.9 ± 0.02 a	2.6 ± 0.04 a
		June 2016	3.7 ± 0.9 ab	2.3 ± 0.03 a	0.9 ± 0.01a	2.7 ± 0.03 a
		July 2016	1.5 ± 0.8 b	2.1 ± 0.03 b	0.8 ± 0.01 b	2.7 ± 0.03 a
	UAFRS	Sept. 2015	*	2.2 ± 0.02 c	0.9 ± 0.01 bc	2.6 ± 0.03 ab
		Oct. 2015	*	2.5 ± 0.03 a	0.9 ± 0.02 a	2.7 ± 0.03 a
		May 2016	*	2.5 ± 0.07 ab	0.9 ± 0.05 ab	2.7 ± 0.08 ab
		June 2016	*	2.3 ± 0.03 bc	0.9 ± 0.01 a	2.6 ± 0.03 b
		July 2016	*	2.1 ± 0.03 d	0.8 ± 0.01 c	2.6 ± 0.03 ab
		Aug. 2016	*	2.1 ± 0.03 d	0.8 ± 0.01 c	2.6 ± 0.03 ab
2017	UAFRS	May	4.0 ± 0.9 a	2.8 ± 0.05 c	1.0 ± 0.02 bc	2.8 ± 0.05 a
		June	3.6 ± 0.9 a	2.8 ± 0.05 c	1.0 ± 0.02 c	2.9 ± 0.05 a
		July	2.0 ± 0.9 a	3.3 ± 0.05 a	1.2 ± 0.02 a	2.8 ± 0.05 a
		Aug.	4.2 ± 0.9 a	3.1 ± 0.05 b	1.1 ± 0.02 b	2.9 ± 0.05 a
	Farm 1	May	11.2 ± 1.4 a	2.9 ± 0.08 cd	1.0 ± 0.04 bc	2.8 ± 0.07 a
		June	5.1 ± 0.8 b	2.8 ± 0.04 d	1.0 ± 0.02 c	2.9 ± 0.04 a
		July	2.3 ± 1.0 b	3.2 ± 0.05 ab	1.1 ± 0.02 ab	2.9 ± 0.05 a
		Aug.	4.1 ± 1.0 b	3.1 ± 0.05 bc	1.1 ± 0.02 ab	2.8 ± 0.05 a
		Oct.	12.6 ± 1.5 a	3.4 ± 0.08 a	1.2 ± 0.04 a	2.8 ± 0.08 a

Mean values within a column followed by the same letter are not significantly different ($P > 0.05$; Tukey's HSD).
*Due to long storage of flies in 70% ethanol, the pigmentation could not be scored.

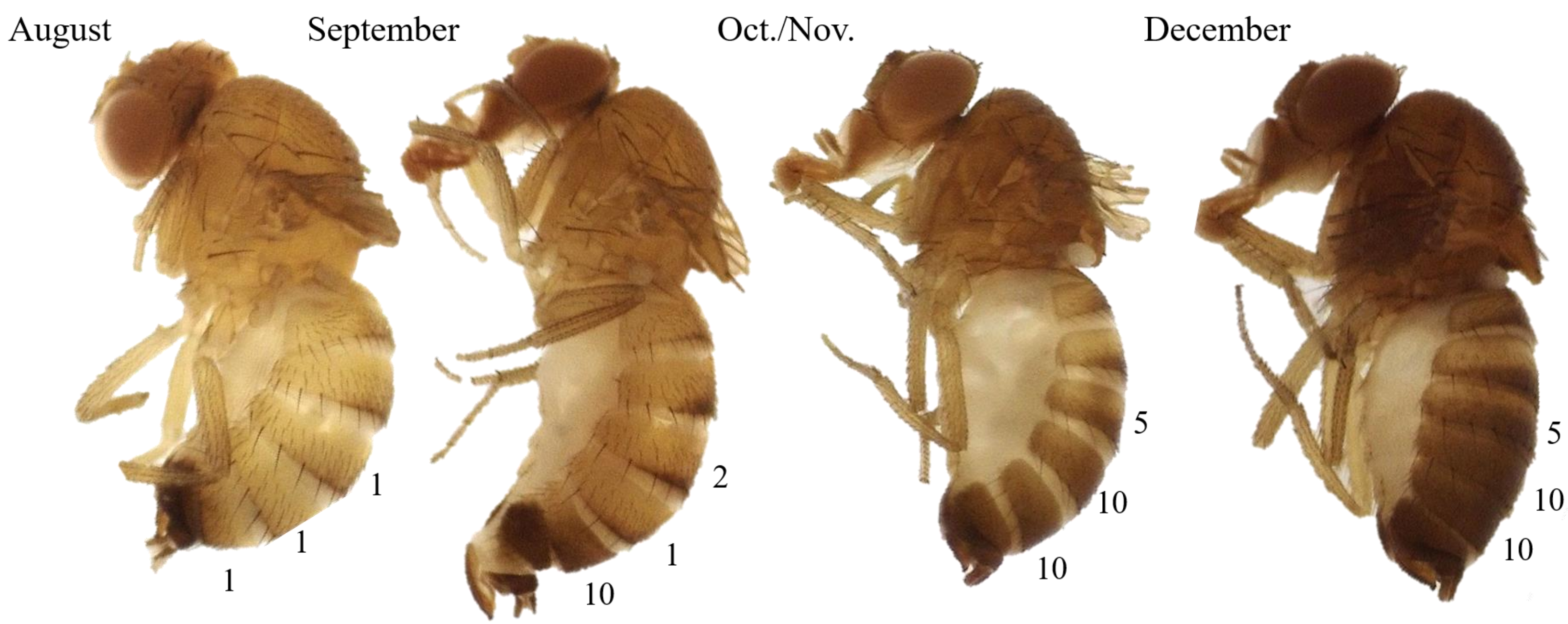

Figure 5: Female spotted wing drosophila tergite pigmentation varying by months at Nashville, Tennessee (2013).

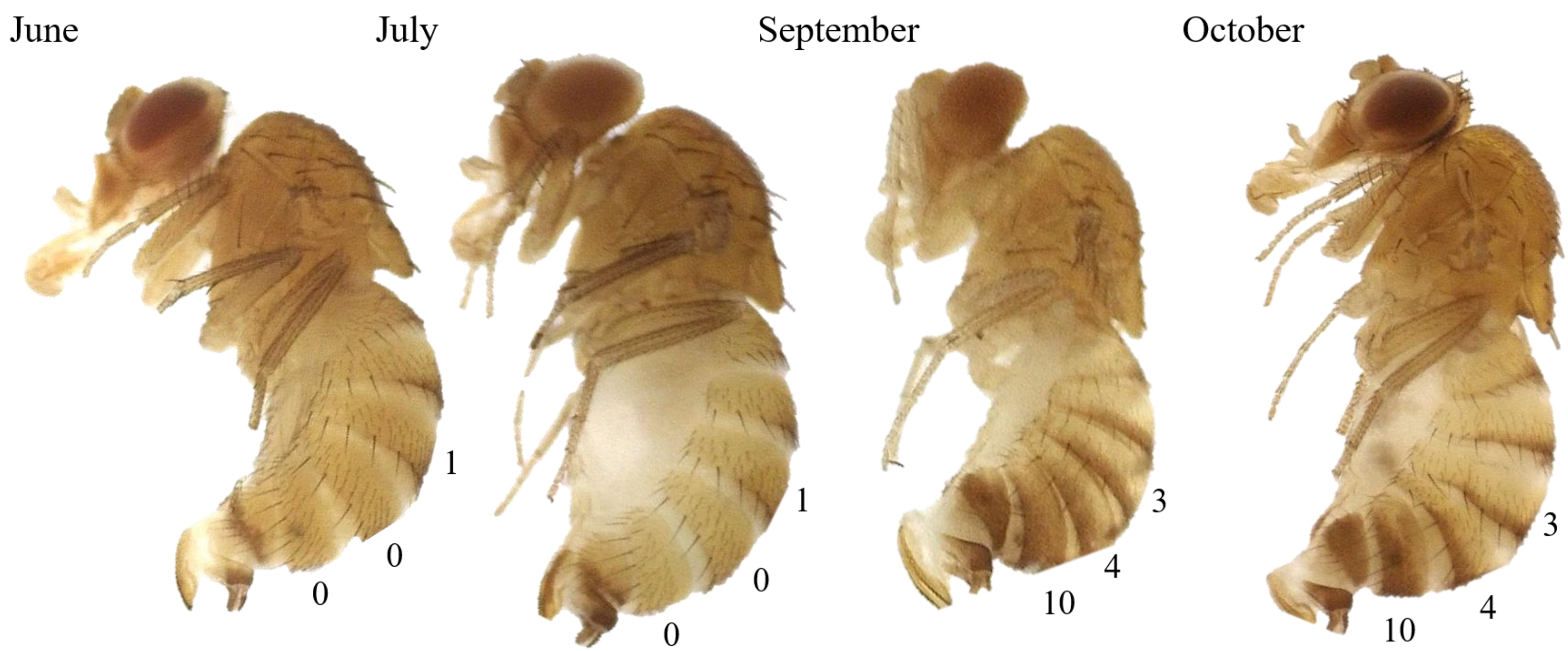

Figure 6: Female spotted wing drosophila tergite pigmentation varying by months at Arkansas Agricultural Research and Extension Center farm in Fayetteville, Arkansas (2015-2016).

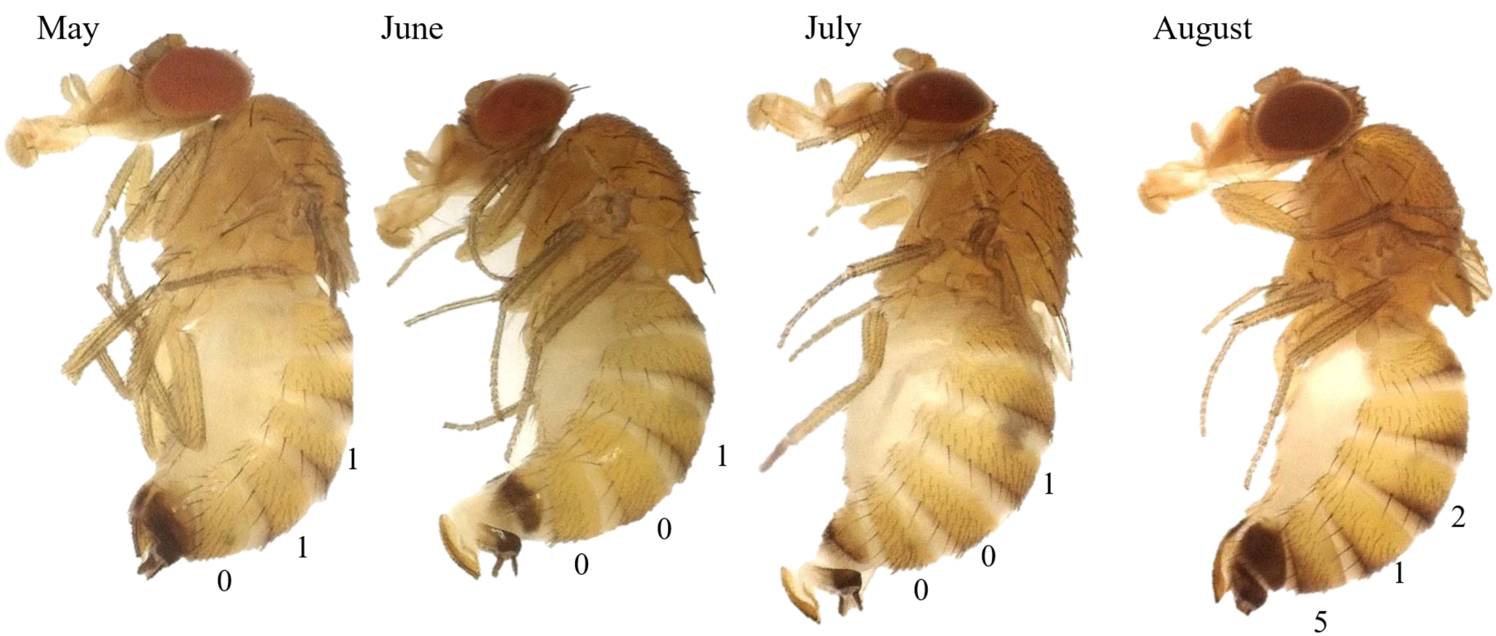

Figure 7: Female spotted wing drosophila tergite pigmentation varying by months at the University of Arknsas System Division of Agriculture Fruit Research Station in Clarksville, Arkansas (2017).

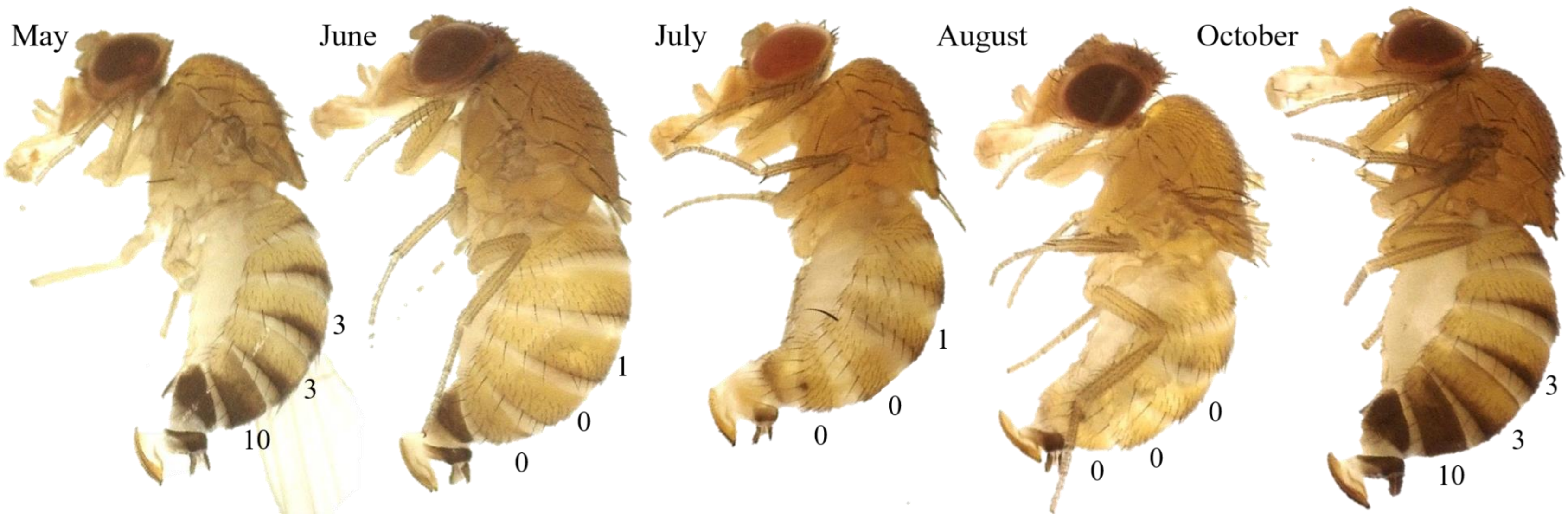

Figure 8: Female spotted wing drosophila tergite pigmentation varying by months at private farm near Tontitown, AR (2017).

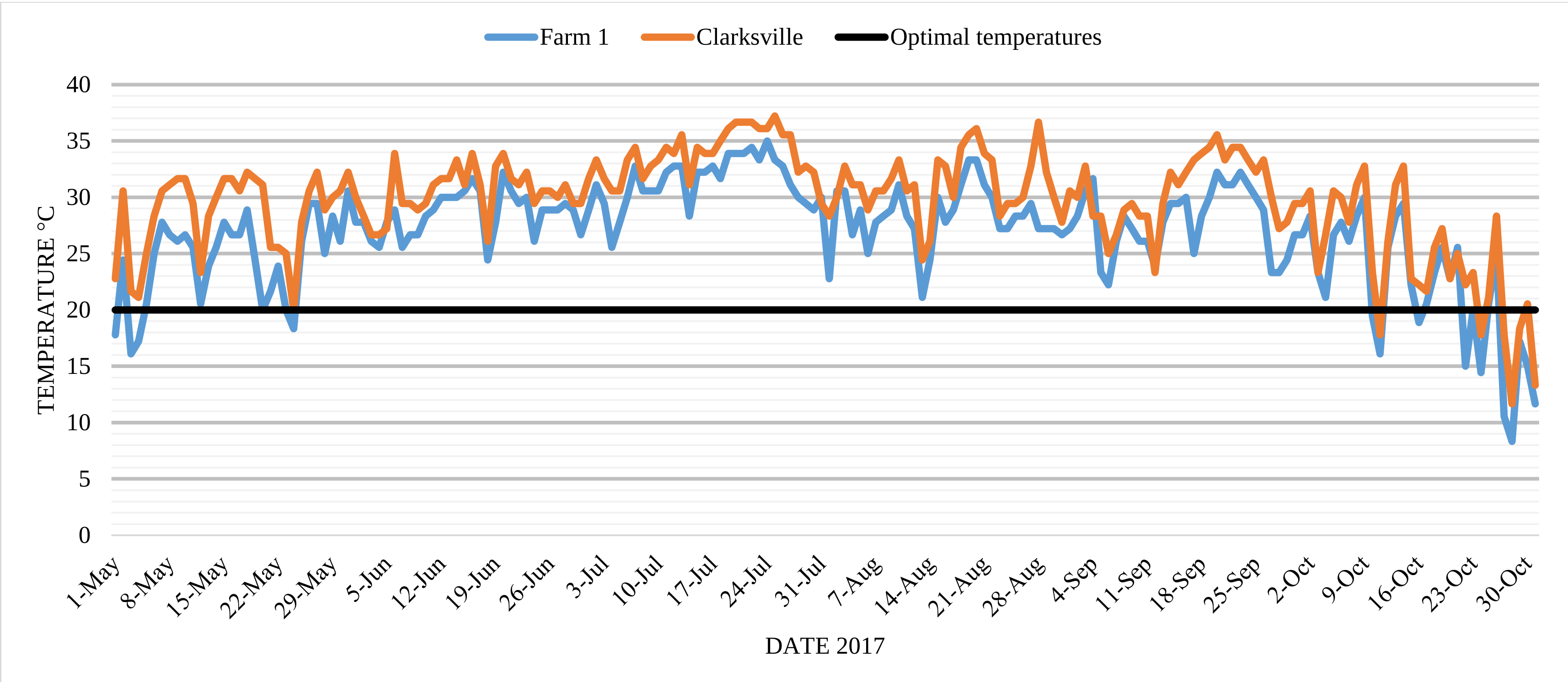

Figure 9: Maximum daily temperatures in Arkansas at the private farm (Farm 1) near Tontitown and University of Arkansas System Division of Fruit Research Station in Clarksville from May-October 2017 (online phenology and degree-day model site uspest.org 2017). Spotted wing drosophila can be active when temperatures exceed 20°C (black line).

Chapter 3: Overwintering and Alternative Hosts of SWD

Abstract

Drosophila suzukii (Matsumura), or spotted wing drosophila (SWD), was introduced into the United States in 2008 and has become a worldwide pest of soft-bodied fruit crops. While much has been studied to manage SWD, it remains unknown how the adults survive the winter with limited food resources and colder temperatures. Although most susceptible fruit production occurs during summer months with ideal temperatures for SWD, many non-cultivated fruits can be found within or adjacent to a grower's fields, providing alternative food sources during late fall, winter, and early spring. It is also common for the ratio of adult male to female SWD to fluctuate throughout the year. Female numbers are usually higher than males towards the end of fall and early winter. Females are usually the first SWD caught at the beginning of the year suggesting that they have survived colder temperatures better than males. Early-emerging females lay the first generation of eggs for the new year. The objective in this study was to determine when SWD are active during the winter months in Arkansas and what potential alternative hosts they might utilize. Traps were placed at two locations in Fayetteville, AR (Arkansas Agricultural Research and Extension Center and Wilson Park) to monitor adult SWD activity from October 2017 to May 2018. Potential alternative hosts were collected in these areas to determine the wild hosts SWD might be utilizing during the winter. Trap catches of SWD adults were high (>100 per month) until early January 2018 (three flies caught). In December, female SWD became more abundant than males. The first wild host fruit that SWD eggs were found in was mulberry (*Morus rubra* L.) in early June. Mulberry ripens in late-May to early June in Arkansas providing a food source for SWD before cultivated crops, such as blackberries, ripen. Carolina buckthorn (*Rhamnus caroliniana* (Walter) A. Gray) and bush honeysuckle

(*Lonicera maackii* (Rupr.) Herder) began to ripen towards the end of blackberry harvest and fruits remained available into December. The late season activity of SWD indicates that they can find the necessary resources to survive winter conditions.

Keywords: spotted wing drosophila, alternative host, overwintering

Introduction

Drosophila suzukii (Matsumura) or spotted wing drosophila (SWD) rapidly became a widespread pest of soft-bodied fruit crops throughout the United States since being introduced in 2008 (Hauser 2011). Unlike most *Drosophila* species, that are attracted to overripe or spoiled fruit, SWD is attracted to intact nearly ripe or ripe soft-skinned fruit (Cini et al. 2012; Asplen et al. 2015). Female SWD have a serrated ovipositor that allows them to attack both ripening and ripe fruit (Hauser 2011; Wash et al. 2011; Asplen et al. 2015). In 2008, a yield loss of 20% resulted in millions of dollars lost to farmers of strawberries and blueberries in California, Oregon and Washington (Lee et. al 2011b). SWD have a wide host range, including many alternative hosts (non-crop hosts) (Cini et al. 2012; Lee et al. 2015). Susceptibility of a fruit to SWD seems to be influenced by color, odor, texture, firmness and size (Lee et al. 2011a). Fruit such as peaches are not suitable for SWD because its fuzzy surface prevents oviposition (Stewart et al. 2014). Host preference for female SWD seems to depend on the local abundance of the hosts (Cini et al. 2012). Known alternative hosts include: honeysuckle (*Lonicera* spp.), autumn olive (*Elaeagnus umbellata* Thunberg), pokeweed (*Phytolacca americana* L.), and mulberry (*Morus* spp.) (Lee et al. 2015). Fruit of plants found within the families of Adoxaceae, Beberidaceae, Buxaceae, Caprifoliaceae, Cornaceae, Elaeagnaceae, Lauraceae, Moraceae, Phytolaccaceae, Thamnaceae, Rosaceae, and Solanaceae were found to be susceptible to SWD (Lee et al. 2015).

Alternative hosts have been noted to help sustain SWD into the winter months (Cini et al. 2012, Lee et al. 2015). However, it is still unclear how and where SWD overwinter. Other *Drosophila* species are known to overwinter in man-made shelters or under leaf litter and SWD may exhibit a similar behavior (Stephen et al. 2015; Wallingford and Loeb 2016). A study in

Wisconsin and Minnesota found that farms surrounded by larger areas of woodland, on average, caught flies in traps one week earlier than farms not heavily surrounded by woodland suggesting that more resources (alternative hosts) were available in those areas (Pelton et al. 2016). In Italy, adult SWD were caught continuously throughout the winter in some locations but not in others, suggesting that some habitats may be less suitable for overwintering (Rossi-Stacconi et al. 2016). Kaçar et al. (2016) found that SWD must acquire nutrients from a food source to meet energetic and metabolic requirements necessary to survive through the winter. In Oregon, SWD were observed feeding on damaged persimmons, figs and rotting apples from October to January (Lee et al. 2015). Not only do alternative hosts play a role in sustaining SWD, but adults have been known to feed on sap from wounded oak trees as well (Lee et al. 2015). These food resources can contribute to higher densities of SWD before, during and after a growing season and support SWD development when crop hosts are not available (Lee et al. 2015).

Along with discovering where SWD overwinter, it is also important to understand how adults survive the cold winter months. A study done by Dalton et al. (2011) indicated that individuals should not be able to survive in the winter months due to the prolonged cold weather. In the Pacific Northwest, if temperatures were consistently below 11.6°C for 109 days, 50% mortality of SWD is expected (Coop 2011). The sex ratio of male:female SWD changes throughout the year and can provide us with information if males and females both overwinter (Rossi-Stacconi et al. 2016). A higher ratio of males in the late spring and fall and more females in the summer has been observed (Dalton et al. 2011; Rossi-Stacconi et al. 2016). Females are the majority of flies caught during the first part of a new year when temperatures are cooler, which suggests that female SWD are overwintering (Dalton et al. 2011; Rossi-Stacconi et al. 2016). Female SWD have been found to survive longer than males at colder temperatures

(Plantamp et al. 2016; Shearer et al. 2016). Kimura (2004) found the low lethal temperatures able to kill half of a SWD population (LT_{50}) to be -1.6°C and -0.3°C for both females and males, respectively. When returned to 20°C, SWD female flies that were exposed to cold temperatures produced a similar number of offspring compared to females that were not exposed to cold temperatures (Plantamp et al. 2016). Mated females are considered to be the overwintering life stage of SWD (Kanzawa, 1939, Cini et al. 2012). In some *Drosophila* species male sterility is induced once temperatures reach 12°C or below which may explain why mated females are the overwintering life stage (Dalton et al. 2011). It is important to understand at what life stage SWD is overwintering as and what alternative hosts they may be utilizing to help them overwinter.

Little is known about the overwintering or biology of SWD in Arkansas. This study was conducted in Arkansas to monitor potential host utilization and adult activity during the overwintering period for SWD. Between the fall and spring (2016-2018), potential alternative host fruits were collected, SWD reared from fruits and baited SWD traps (2017-2018) monitored for SWD activity.

Materials and Methods

Monitoring

This study was conducted at two locations in Fayetteville, AR: the Arkansas Agricultural Research and Extension Center (AAREC) **(Fig. 1A)** and Wilson Park **(Fig. 1B)**. At each location two traps for adult SWD were placed in areas suitable for SWD. At the AAREC traps were placed near potential hosts such as bush honeysuckle (*L. maackii*), pokeweed (*P. americana*) and cultivated blackberry (*Rubus* spp.) (36°5'N, -94°10'W; 36°5'N, -94°9'W). Due to the high human activity at Wilson Park, SWD traps were placed in a small grove of trees where potential hosts such as pokeweed (*P. americana*), bush honeysuckle (*L. maackii*) and mulberry (*Morus* *rubra* L.) were present nearby (36°04'N, -94°09'W). SWD traps were monitored every other week from October 2017 to May 2018. Pherocon® (Trėcė incorporated, Adair, OK) traps were baited with a Trėcė SWD three component high specificity lure and apple cider vinegar that acted both as an attractant and a drowning solution (**Fig. 2A-B**). Minimum and maximum temperatures were documented during the trapping period to see if the temperature affected trap counts.

Alternative hosts

During the years of 2016 to 2018, fruits from potential alternative hosts for SWD were collected, identified to species when possible and examined for SWD eggs. Plants were identified to species with two experts (personal communication with Dr. Garry McDonald, Clinical Horticulture Assistant Professor, and Dr. Don Steinkraus, Professor of Entomology, University of Arkansas and referred to keys by Hunter, 1995). Many of the hosts were collected from Fayetteville, AR with a few from Cassville, MO, ranging from parks, trails and other areas with wild plants. Thirty ripening to ripe fruits were collected from each plant species and examined using a dissecting microscope to determine presence of SWD eggs or larvae. Each infested fruit was then placed into a Solo® 37 ml cup (Lake Forest, IL) with a moist cotton ball if needed **(Fig. 3)** to check for adult SWD emergence. Cups were held at 21°C with about 60-70% humidity for one month to record adult emergence. Adult emergence is proof that the sampled host supported the SWD life cycle.

Results

In October and November 2017, SWD adults were caught in high numbers (average 40 flies or more per trap) **(Fig. 4)**. At the end of December and early January (Dec. 24-Jan.-6) **(Fig. 7)**, minimum ambient temperatures fell below the LT_{50} (1.6°C), which resulted in fewer flies caught during that period **(Fig. 4-6)**. Fly catch eventually reached zero in early January at Wilson Park then a few weeks later at the AAREC location **(Fig. 4-6)**.

Eight different plant species were shown to be alternative hosts for SWD in Northwest Arkansas and Southwestern Missouri **(Table 1)**. Adult SWD emerged from ripe fruit of seven of these alternative hosts. In 2016 and 2017, beauty berry (purple) (*Callicarpa americana* L.) fruits contained SWD eggs but no adults emerged from the samples. In May 2017, mulberry (*M. rubra*) had the first ripe fruit collected, but no eggs were found, and no adults emerged from the fruit. However, a few weeks later in June, at the same location, SWD eggs were found in mulberry fruits and adult SWD emerged. In 2018, SWD eggs were present in mulberry as early as May 28. Out of the non-cultivated host plants sampled in Arkansas, mulberries were the earliest recording of SWD eggs in a fruit **(Table 2)**. During the summer months of June to August, pokeweed (*P. americana*), trumpet honeysuckle (*L. sempervirens* L.), elderberry (*Sambucus canadensis* (L.) R. Bolli) and autumn olive (*E. umbellate*) ripen **(Table 2)**. Many of these alternate fruits ripened after crop host fruits had been harvested for the season. Bush honeysuckle (*L. maackii*) and beauty berry (*C. americana*) were the only SWD hosts producing ripe fruit into November with SWD eggs present **(Table 2)**. Adult SWD were caught from October to the early December and many of the alternative hosts collected were also found throughout the fall **(Fig. 4, Table 2)**. In addition to the eight-plant species that were found to have SWD eggs present, fruits from many other plant species were examined that never had SWD eggs present **(Table 3)**. From December 2016 to March 2017, privet (*Ligustrum* spp.) and Euonymus (*Euonymus* spp.) were the only two plant species with fruit that potentially could be an alternative host for SWD. However, neither of these two species had fruits containing SWD eggs and no adults emerged from the winter collection of fruits.

The sex-ratio of adult SWD fluctuated at the two locations **(Fig. 5-6).** The number of males caught was higher than females at both locations from October through November **(Fig. 5-6)**. In December the ratio of males to females began to transition to a higher number of females compared to males until only females were caught in January **(Fig. 5-6)**.

Discussion

Kimura (2004) found the LT_{50} to be -1.6°C for females. In Fayetteville, AR, the longest period of low temperatures at or below the LT_{50} for SWD females occurred for 14 days from 24 December 2017 to 6 January 2018 with the average temperature being -7.4°C **(Fig. 7)**. Even though the daily low temperatures were below the LT_{50}, the average high temperature for that time frame was 0.92°C. After the 14-day period of low temperatures, the average low temperature increased to -3°C for the remainder of January. Dalton et al. (2011) found that acclimated SWD can survive up to 88 days at a constant temperature of 10°C. In the Pacific Northwest, 20% mortality is expected of SWD with 56 days below 11.6°C (Coop 2011). In Arkansas from October 2017- May 2018, the longest stretch of high temperatures below 11.6°C was 17 days from 24 December 2017 to 9 January 2018 **(Fig. 7)**. If SWD found a refuge, they may have been able to survive the cold period. Since adults were found after that period of time, it is likely that there were refuges for female SWD from the lethal cold temperatures. A SWD can survive below-zero temperatures, it suggests that they may rely on a freeze avoidance mechanism which maintains body fluids as liquids and keeps them from freezing (Sinclair et al. 2003; Wallingford et al. 2016). Female SWD flies were caught at the end of January, suggesting they are the overwintering stage in Arkansas. This agrees with other reports that mated females are the overwintering life stage of SWD (Cini et al. 2012, Rossi-Stacconi et al. 2016).

There were four days in January and February that reached 20°C or higher **(Fig. 7)**. Peak activity for adult SWD happens when temperatures are between 20-25°C (Kanzawa 1939, Cini et al. 2012). This suggests that SWD can be active in the winter months here in Arkansas. Dalton et al. (2011) and Rossi-Stacconi et al. (2016) both found that males were more numerous than females during the fall months and that females were the first adults to be caught in the spring. At both the AAREC and Wilson Park in Fayetteville, Arkansas, males were more numerous in October through November than females. It wasn't until December that more females were caught than males **(Fig. 5-6)**. However, it is still unclear how and where they overwinter. Although it has been suggested that the SWD flies find shelter under leaf litter or snow (Stephen et al. 2015; Wallingford and Loeb 2016), it is unlikely that SWD find shelter under snow in Arkansas. In the past two years (2016 and 2017), Arkansas has had little or no snow for SWD to find shelter under. However, they could find shelter indoors or under leaf litter.

In January and February, food resources for SWD were very limited which may explain why no flies were caught in February even though temperatures were favorable on some days. No adults were caught during March-May of 2018. Temperatures were favorable, but there were limited food resources available. In northwest Arkansas during January-April, there are no known alternative hosts that SWD could utilize. By the end of May, mulberry starts to ripen. When mulberries were collected at the end of May, SWD eggs were found, and one adult fly was also reared from the mulberries. This was the first indication of SWD activity in the spring of 2018. Even though adults were not caught in the traps, females were active in late May since eggs were found in mulberry at Wilson Park. The traps were not located near the mulberry tree in Wilson Park due to heavy human activity, which may play a role as to why no adults were caught in the traps.

Herrera (2017) also found that pokeweed (*P. americana*), Carolina buckthorn (*F. caroliniana*), honeysuckle (*Lonicera* spp.) and mulberry (*M. rubra*) to be alternative hosts for SWD in Arkansas. Other species such as autumn olive (*E. umbellate*) and elderberry (*S. canadensis*) were noted to have eggs present in the fruit but no adults emerged (Herrera 2017). However, in this survey eggs were found in both species and adult emergence was observed. These alternative hosts may explain why SWD were caught in high numbers (averaging 40 or more per trap) into December. Only one of the eight host species infested with SWD eggs lacked adult SWD emergence, which may be attributed cold exposure in the field or inadequate rearing method in the laboratory **(Table 1)**. Although adults never emerged from beauty berry (*C. americana*), the presence of SWD eggs found in that sample along with bush honeysuckle (*L. maackii*) provided documentation of SWD activity in November. Adults from bush honeysuckle emerged from fruits brought back into the laboratory suggesting it is possible that there were newly emerged adults in November. In early spring, mulberry was the only alternative host found to be infested with SWD. The only other crop that is ripe early in the year in Arkansas are strawberries. They are ripe in April and last through May to early June and do not get infested with SWD. Since SWD do not infest strawberries, it suggests that the adult flies do not become active until May. Mulberries in Arkansas ripen in May-June, which could provide an alternative food source until cultivated crops, such as blackberries, begin to ripen in June. The ripening periods of SWD alternative hosts sampled were staggered enough to maintain high populations of SWD throughout the summer and fall. **(Table 2)**. Any damaged or overripe fruit can provide food for SWD into the winter (Kaçar et al. 2016). Pelton et al. (2016) noted that farms with wooded areas surrounding them had adult SWD present in traps earlier than those without. One reason SWD are caught earlier is due to possible alternative hosts in that wooded area providing food sources in the winter (Pelton et al. 2016).

Understanding how long SWD is active into the winter months and what alternative SWD hosts exist adjacent to susceptible berry crops can aid in managing this pest. Even though temperatures may dip below the LT_{50} for SWD females, if the high temperature for the day rises above it, SWD can survive those colder periods. It will be important to do more research to see if the winters do get cold enough in Arkansas to kill off part of the populations similar to what Dalton et al. (2011) found in the Pacific Northwest. Finding refuges and utilizing alternative host plant resources allow adult SWD to survive during the Arkansas winter. With alternate hosts, a population of SWD can remain near a susceptible berry crop field after the season is over, increasing the chances of overwintering populations to emerge nearby in the spring. Growers can anticipate an SWD infestation sooner if they have alternate hosts near their fields, especially since SWD females are the first to emerge in the spring. They can set up SWD traps around the perimeters of their fields and monitor for first activity of SWD. Setting out a trap that is effective in catching SWD is crucial for pest management (Lasa et al. 2017). Recently it was found that a trap with red and black stripes, Apple cider vinegar + 10% ethanol and a fermenting sugar-yeast combination placed in a domed shape trap collects more SWD than other traps (Lasa et al. 2017). When they start to catch adults in the traps, they can then take action to combat this pest dispersing to a susceptible ripening berry crop.

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Appendix

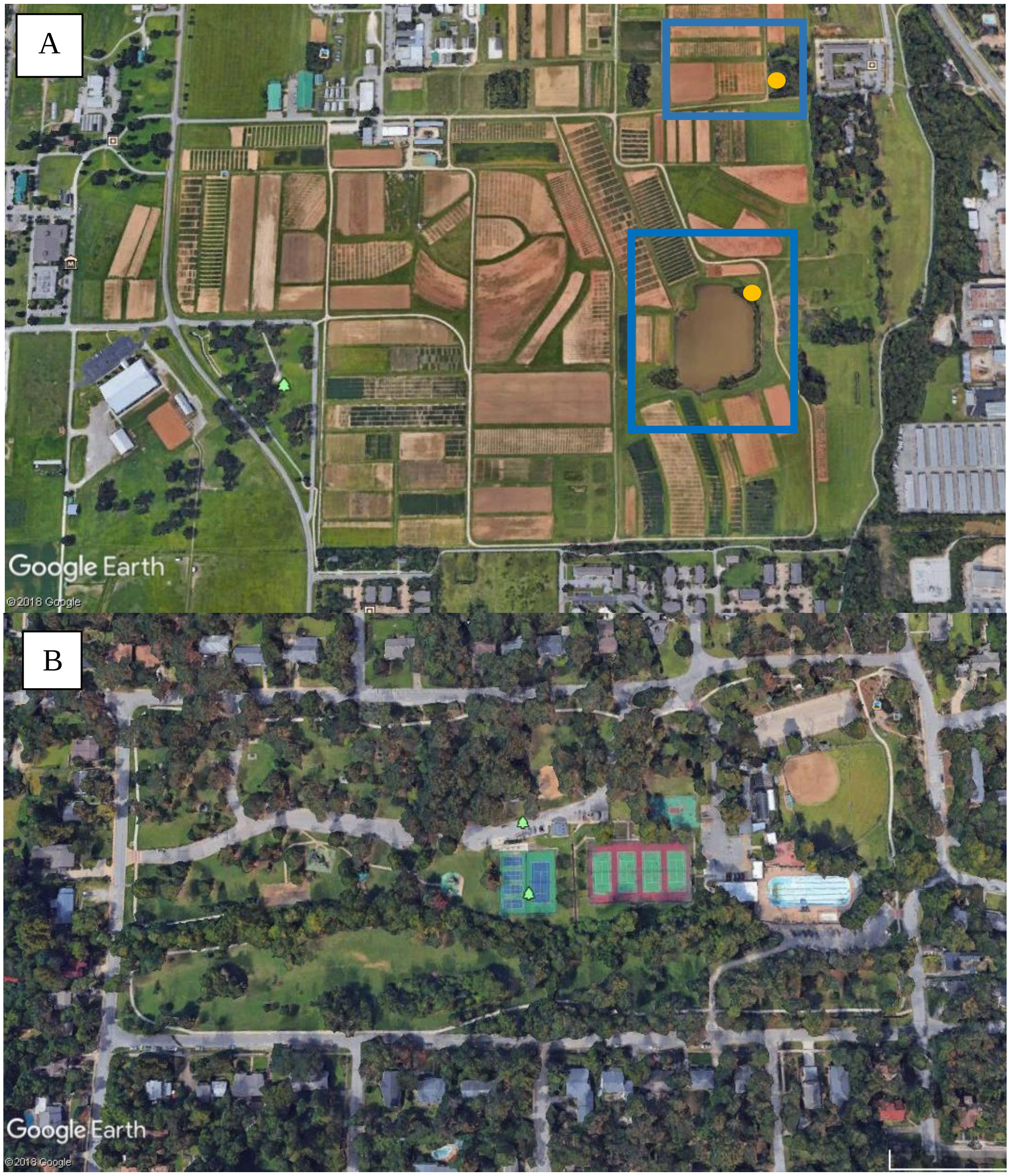

Figure 1: Trap locations (orange dot) that contained potential alternative hosts for spotted wing drosophila at A) Arkansas Agriculture Research and Extension and B) Wilson Park, in Fayetteville, AR.

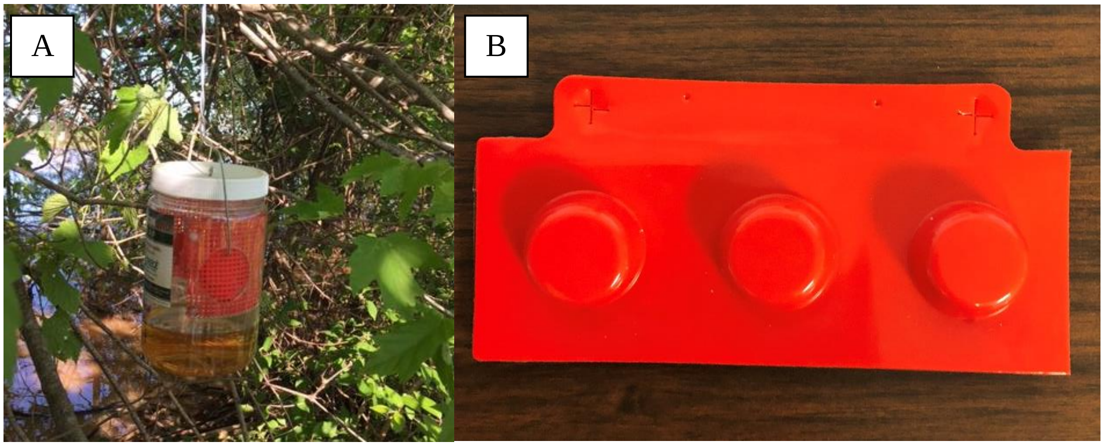

Figure 2: Spotted wing drosophila (SWD) Trėcė trap containing: A) attractive apple cider vinegar drowning solution and B) three component Trėcė SWD lure placed inside trap.

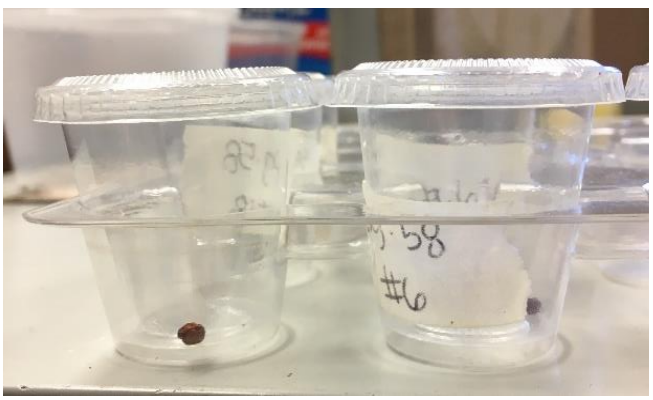

Figure 3: Individual 37 ml Solo® cups containing spotted wing drosophila infested fruit in the laboratory.

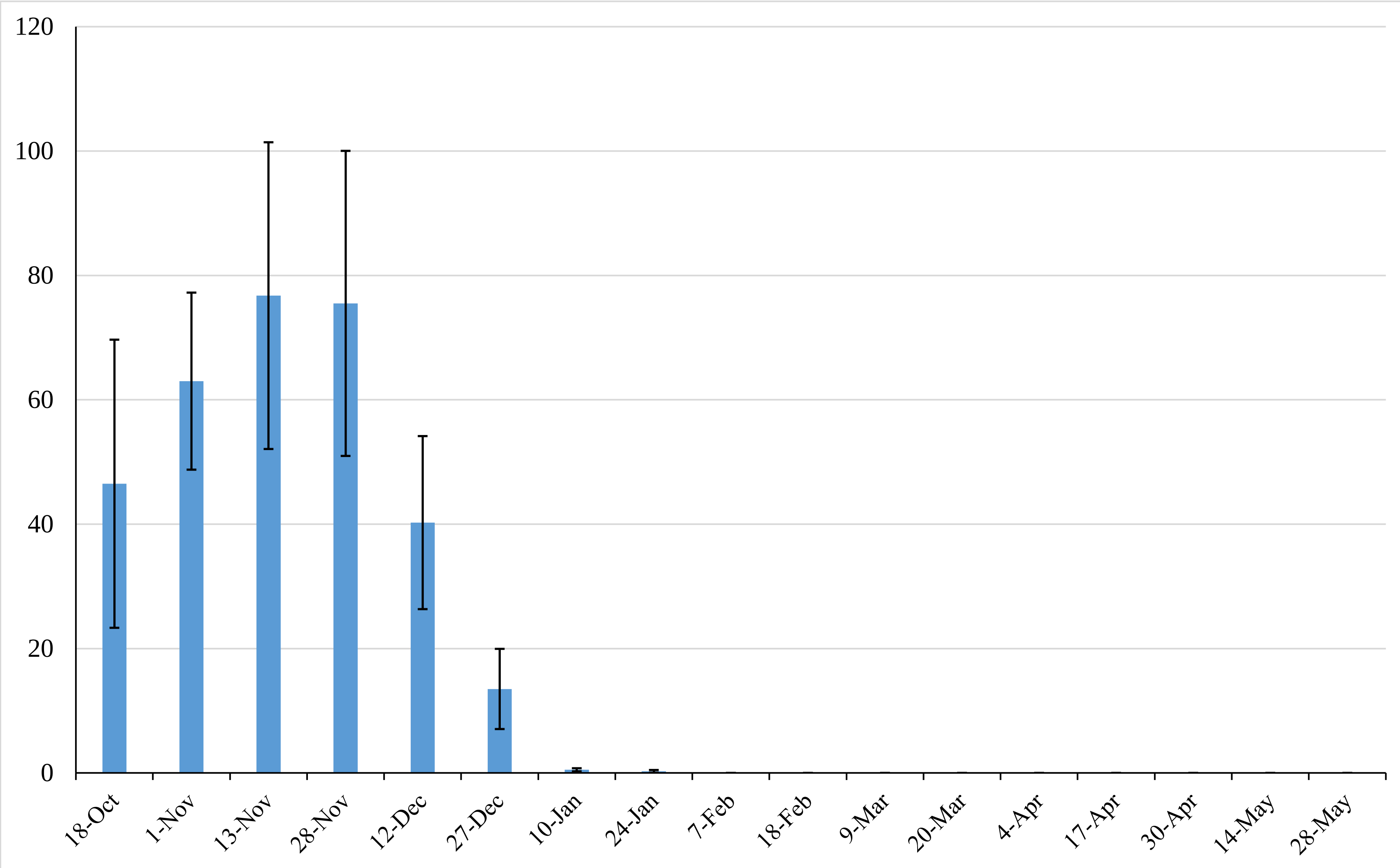

Figure 4: The mean number (±SE) of spotted wing drosophila adults caught per trap baited with Trėcė three component lure and apple cider vinegar at both (combined data) the Arkansas Agriculture Research and Extension Center and Wilson Park (Fayetteville, AR) from October 2017-May 2018.

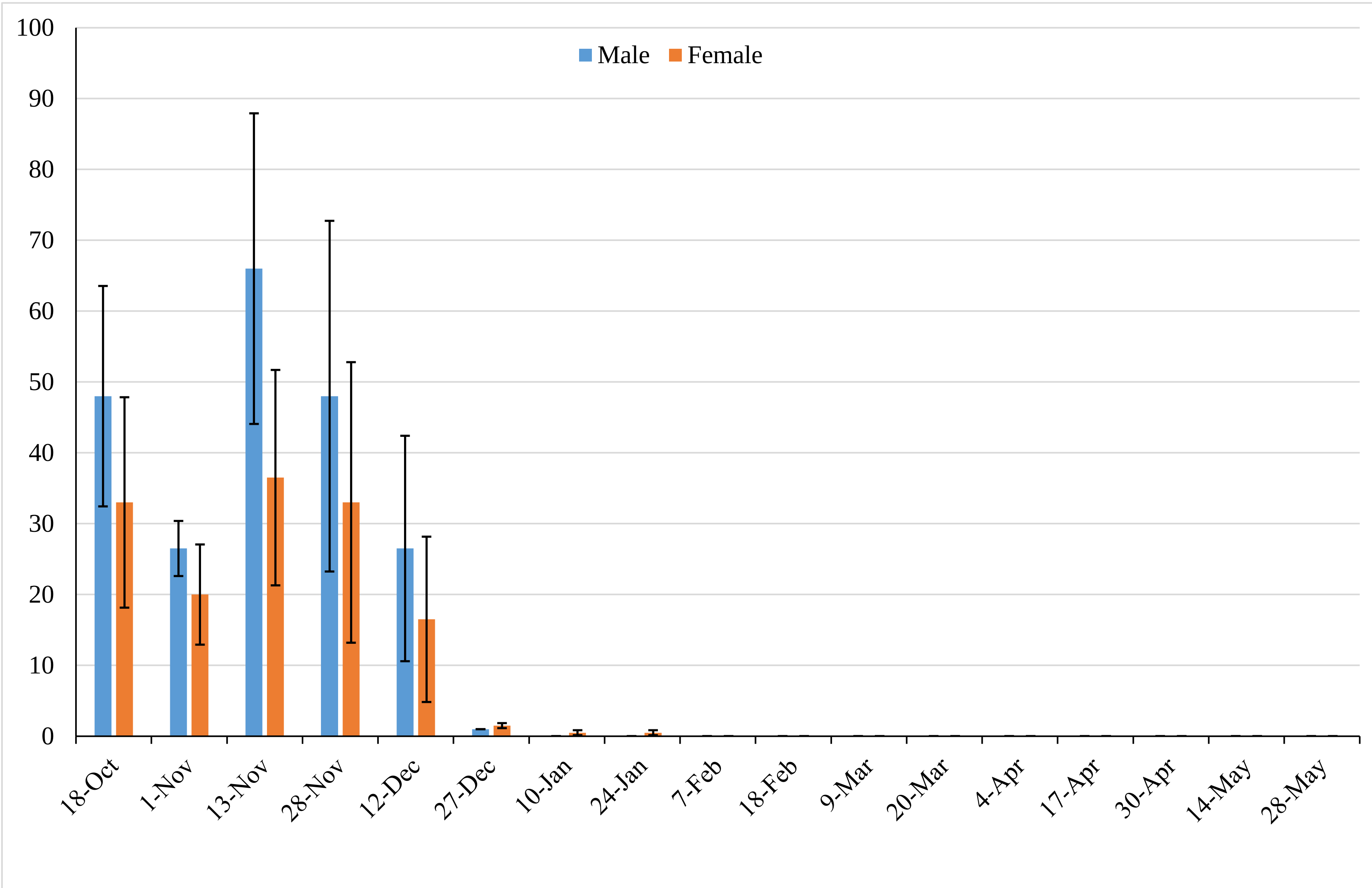

Figure 5: Mean number (±SE) of male and female spotted wing drosophila adults per trap baited with Trėcė three component lure and apple cider vinegar at the Arkansas Agricultural Research and Extension Center in Fayetteville, AR from October 2017-May 2018.

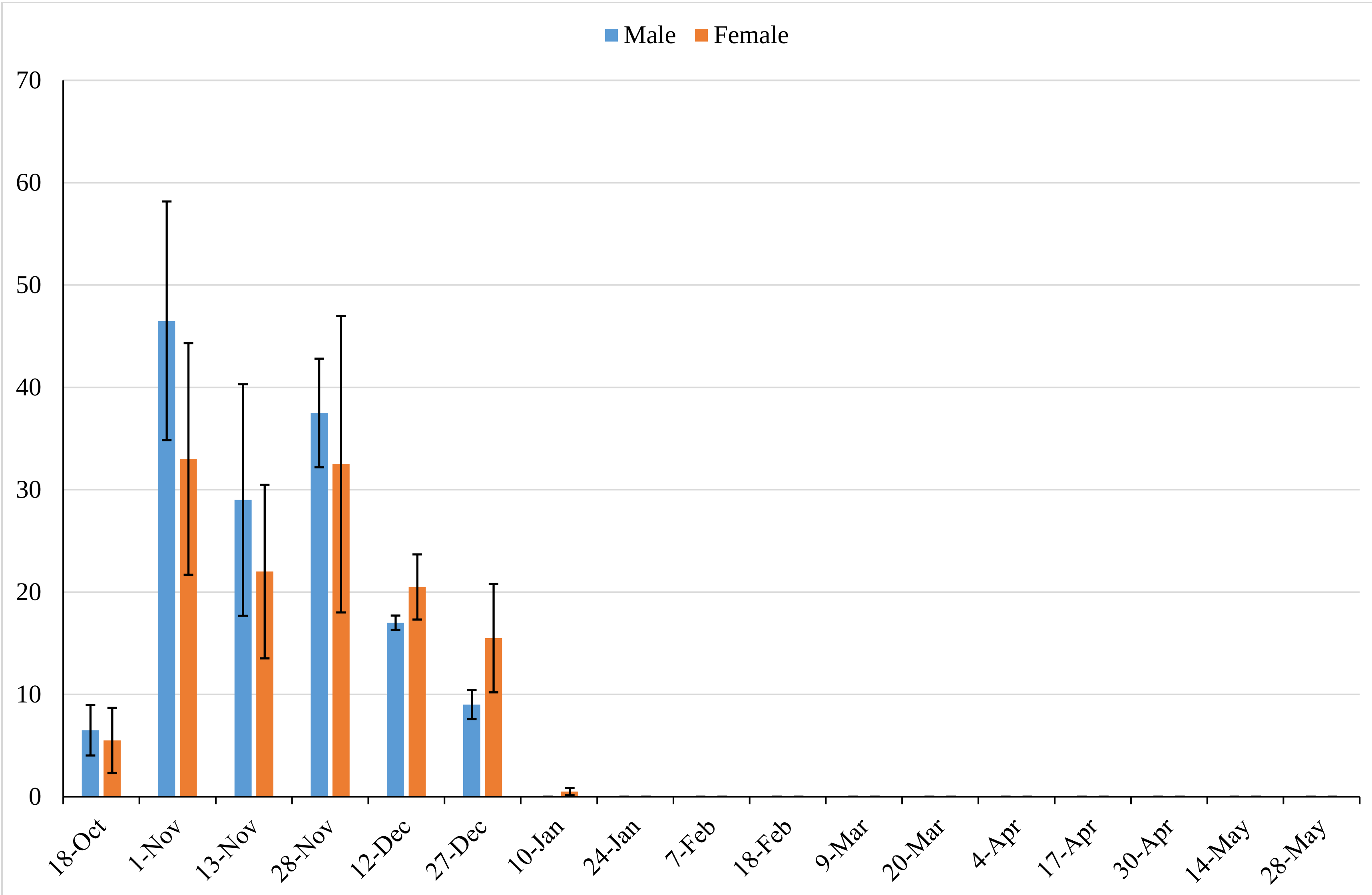

Figure 6: Mean number (±SE) of male and female spotted wing drosophila adults per trap baited with Trėcė three component lure and apple cider vinegar at Wilson Park in Fayetteville, AR from October 2017 - May 2018.

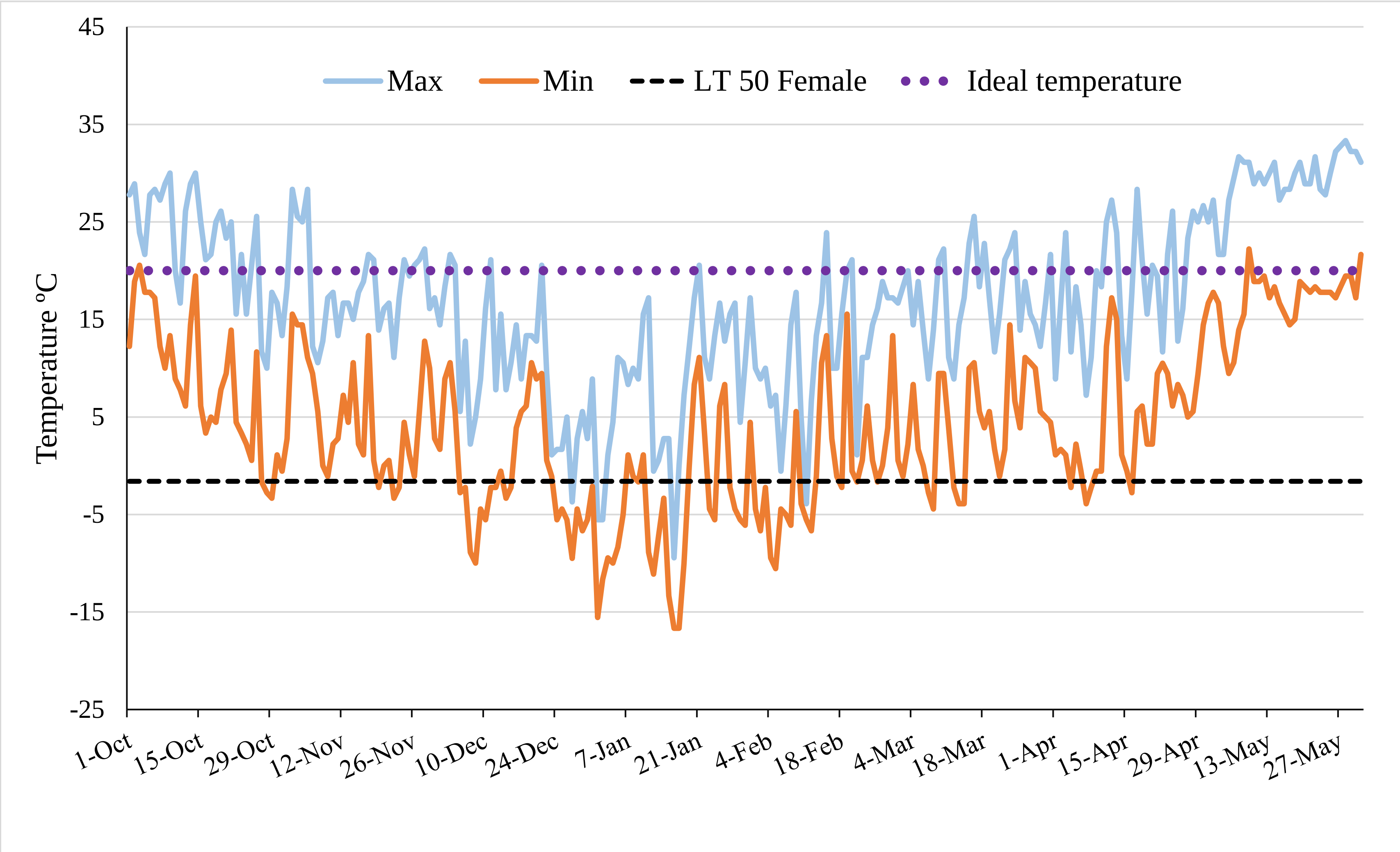

Figure 7: Maximum and minimum temperatures of Fayetteville, Arkansas from October 1, 2017-May 31, 2018 with lethal temperature killing 50% of the population (LT_{50}) of spotted wing drosophila (SWD) females (-1.6°C) and ideal temperature (20°C) when conditions start to be favorable for SWD to become active (online phenology and degree-day model site uspest.org 2017-2018).

Table 1: List of alternative plant hosts that spotted wing drosophila (SWD) females laid eggs in and adults emerged from July 2016-June 2018 in Fayetteville, AR and Cassville, MO.

Family	Scientific Name	Common Name
Caprifoliaceae	*Sambucus canadensis* (L.) R. Bolli	Elderberry
	Lonicera maackii (Rupr.) Herder	Busch honeysuckle
	Lonicera sempervirens L.	Trumpet honeysuckle
Elaeagnaceae	*Elaeagnus umbellata* Thunb.	Autumn olive[+]
Moraceae	*Morus rubra* L.	Red mulberry
Phytolaccaceae	*Phytolacca americana* L.	Pokeweed[0]
Rhamnaceae	*Rhamnus caroliniana* (Walter) A. Gray	Carolina buckthorn
Verenaceae	*Callicarpa americana* L.	Beauty berry (purple)*

[+]Found only in Cassville, MO
[0]Only plant found at both locations
*No SWD adult emergence

Table 2: List of ripening periods of known alternative hosts for spotted wing drosophila (SWD) from July 2016-June 2018 in Fayetteville, AR and Cassville, MO.

	May	**June**	**July**	**August**	**September**	**October**	**November**
Mulberry (*Morus rubra* L.)	■	■					
[0]Pokeweed (*Phytolacca americana* L.)			■	■	■	■	
Trumpet honeysuckle (*Lonicera sempervirens* L.)			■	■	■	■	
Carolina buckthorn (*Rhamnus caroliniana* (Walter) A. Gray)				■	■	■	
Elderberry (*Sambucus canadensis* (L.) R. Bolli)				■			
[+]Autumn Olive (*Elaeagnus umbellate* Thunb.)				■			
Bush honeysuckle (*Lonicera maackii* (Rupr.) Herder)					■	■	■
*Beauty berry (purple) (*Callicarpa americana* L.)							■

[+]Found only in Cassville, MO
[0]Only plant found at both locations
*No SWD adult emergence

Table 3: List of potential alternative plant hosts that had no spotted wing drosophila eggs or adult emergence from July 2016-June 2018 in Fayetteville, AR.

Family	Scientific Name	Common Name
Anacardiaceae	*Rhus glabra* L.	Smooth sumac
	Rhus copallinum L.	Winged sumac
Annonaceae	*Asimina triloba* (L.) Dunal	Paw paw
Aquifoliaceae	*Ilex sp.*	Holly
Araceae	*Arisaema dracontium* (L.) Schott	Green Dragon
Araliaceae	*Aralia spinosa* L.	Devil's walkingstick
Asparagaceae	*Asparagus officinalis* L.	Asparagus
Caprifoliaceae	*Symphoricarpos orbiculaus* Moench	Buckbrush/Coral berry
Celastraceae	*Euonymus sp.*	Euonymus
Cornaceae	*Cornus florida* L.	Flowering dogwood
Cupressaceae	*Juniperus virginiana* L.	Eastern red cedar
Ebenaceae	*Diospyros virginiana* L.	Persimmon
Lauraceae	*Lindera benzoin* (L.) Blume	Spicebush*
Moraceae	*Maclura pomifera* (Raf.) C.K. Schneid	Osage orange
Oleaceae	*Ligustrum sp.*	Privet
Passifloraceae	*Passiflora lutea* L.	Yellow passion flower
	Passiflora incarnata L.	Passion flower
Rosaceae	*Prunus serotina* Ehrh.	Black cherry
	Rosa multiflora Thunb.	Multiflora rose
	Pyrus sp.	Pear
	Pyrus angustifolia (Aiton) Michx.	Native crabapple
Solanaceae	*Solanum carolinense* L.	Horse nettle
	Solanum lycopersicum L.	Tomato
Verenaceae	*Callicarpa americana* L.	Beauty Berry (White)
Vitaceae	*Vitis sp.*	Wild grape

*Also found in Cassville, MO.

Chapter 4: Entomopathogenic Fungi for Management of Spotted Wing Drosophila

Abstract

Drosophila suzukii (Matsumura), or spotted wing drosophila (SWD), is an invasive fruit fly pest that can require some fruit and berry growers to apply an additional 5-8 insecticide sprays during ripening and harvest period each year. It is important for fruit growers to find alternatives that can help control this pest. Several species of entomopathogenic fungi have been studied to see if they can help control SWD. *Beauveria bassiana* can infect more than 700 different species of arthropods and is often found naturally in the soil. In this study, a commercial formulation of *B. bassiana* strain GHA, Mycotrol® WPO, commercially available to growers was used. Adult SWD were sprayed directly with one of four treatments: high = 2.2×10^8 *B. bassiana* spores per ml; medium = 2.2×10^7 *B. bassiana* spores per ml; low = 2.2×10^6 *B. bassiana* spores per ml or water control. The flies were put into containers with food and after one week any dead SWD were removed and placed on filter paper for a week to see if the cause of death was from *B. bassiana* by sporulation. Although there was some *B. bassiana* caused-mortality of adult SWD, most mortality was from other causes. The high treatment had the highest level of infected flies at 1.7% (two flies sporulated; n = 119) followed by low treatment which killed 0.9% (one fly sporulated; n = 117). It was found that there were no significant differences in mortality between the treated flies and the water controls. The contact application of the commercial formulation of *B. bassiana* did infect a few adult SWD, but percentage mortality was too low to provide control of SWD.

Keywords: spotted wing drosophila, *Beauveria bassiana*, entomopathogenic fungi

Introduction

Since the introduction of *Drosophila suzukii* (Matsumura), common name spotted wing drosophila (SWD) to the United States and other countries, fruit growers and scientists have been trying to find methods to control this pest. Female SWD can begin to lay eggs two days after emergence and lay hundreds of eggs during each female's lifespan (Lee et al. 2011). When the temperature is optimum for development between 24 to 26°C SWD can complete its life cycle in 11 days (Kanzawa 1939, Tochen et al. 2014). With such a short generation time, fruit and berry growers are having to make an additional 5-8 insecticide sprays per growing season (Woltz et al. 2014). The potential for development of insecticide resistance with SWD is a concern (Bruck et al. 2011) due to SWD's short generation time, high fecundity and the long ripening and harvest season of their hosts (Gargani et al. 2013, Asplen et al. 2015). Organic growers have limited insecticide options (Woltz et al. 2014) with spinosad (Entrust) being the most effective (Bruck et al. 2011). Organic growers must follow the spinosad label closely and regularly rotate it with one or more pyrethrin applications (Bruck et al. 2011). Due to potential insecticide resistance, it is important that alternative measures are researched.

Researchers have turned to entomopathogens such as nematodes and fungi, as alternatives to insecticides, to determine if they can help control SWD. It is estimated that between 700 and 1000 species of fungi can cause diseases in arthropods (Lacey 2017). There are several dipteran species in different families that are susceptible to entomopathogenic fungi such as *Beauveria bassiana*, *Metarhizium anisopliae* and *Isaria fumosorosea* (Cossentine et al. 2016). Entomopathogenic fungi attack the host through the cuticle with spores attaching to the epicuticle (Lacey 2017).

Many entomopathogenic fungi can be found naturally in the environment. The soil provides protection from UV radiation and extreme temperatures (Lacey 2017). *B. bassiana* is one of the fungi distributed in the soil (Webster and Weber 2007). *B. bassiana* is a well-studied fungus that is known to infect more than 700 different species of insects and mites. It has been used in the control of agricultural and forest pest (Li et al. 2011). It is known to infect *Aedes aegypti* (Diptera: Culicidae) and house flies, *Musca domestica* (Diptera: Muscidae) (Cossentine et al. 2016) and potentially control larvae of *Apriona germari* (Coleoptera: Cerambycidae) (Li et al. 2011). There are differing results in the literature as to how affective *B. bassiana* is in controlling SWD. Woltz et al. (2014) did not find any effects on SWD adult mortality when sprayed with *B. bassiana* strain GHA (Laverlam International Coporation, Bute, MT). Cuthbertson et al. (2014) found that *B. bassiana* (Naturalis 0.3% solution) caused mortality of adult SWD but would not control the fly population since females still laid eggs before they died from infections. In another study conducted in Italy, infested blueberries were immersed in products to see if adults would emerge from treated fruit. The researchers of that study found that *B. bassiana* strain GHA (Botanigard®) and *B. bassiana* strain ATTC74040 (Naturalis®) helped prevent SWD from emerging (Gargani et al. 2013). The objective of this study was to see if direct sprays of *B. bassiana* could be used as a control method for SWD.

Materials and Methods

SWD Rearing

In the summer/fall of 2016 SWD larvae from blackberries (*Rubus spp.*) and honeysuckle (*Lonicera maackii*) fruit were removed and placed into Ward's Drosophila Media (Rochester, NY) that is a dry yeast mixture mixed with an equal part of water. The following year (2017) additional larvae and adults were collected from blackberries (*Rubus spp.*) and mulberry (*Morus* *rubra*) and placed into the existing colony. Four insect cages 28 cm x 28 cm x 28 cm with a plastic frame and <1mm mesh were used to hold the flies **(Fig. 1A)**. On each of the cages two sides were made of plexiglass making it possible to see inside and check on the fly colony. The cages were held in a room at 25°C ± 1°C, 16:8 L: D. Media was added once a week or as needed to keep the colony growing and provide fresh resources for all SWD life stages to utilize. Media was placed in a Fabri-Kal® (Kalamazoo, MI) 473 ml polypropylene deli containers with a lid **(Fig. 1B)**. Deli lids were placed on the containers to prevent the food from drying out and 2cm holes were cut on the sides of the containers allowing SWD to feed and lay eggs **(Fig. 1C)**.

SWD Collection and Handling

Media was placed in a Fabri-Kal® (Kalamazoo, MI) 473 ml polypropylene deli container without a lid for 72 hours in each of the four cages to allow females to lay eggs. After 72 hours, the containers were removed, a lid was placed on top with holes to allow air passage, and any adult flies still in the containers were removed. The containers were held in the same area as the cages were to keep rearing conditions the same. Once the larvae emerged, they were removed from the deli containers and placed into Drosophila vials to pupate **(Fig. 2).** This allowed the handling of the flies to be easier when trying to sort them for the experiment. The pupae were observed until the first adult emerged to age flies. Flies used in the experiment were 1-4 days old.

Handling B. bassiana

The source of *B. bassiana* came from Arbico organics as Mycotrol® WPO (Wettable powder mycoinsecticide) (Oro Valley, AZ) **(Fig. 3)**. The active ingredient is a *B. bassiana* strain GHA at 22%. It contained 4.78 x 10^{-12} grams per spore and 4.4 x 10^{10} spores per gram. For the initial suspension 0.1 gram of Mycotrol® *B. bassiana* was added to 10 ml distilled water. The starting concentration for 0.1 gram was 4.4 x 10^9 spores. Serial dilutions were then made at the concentrations of 4.4 x 10^8 spores, 4.4 x 10^7 spores and 4.4 x 10^6 spores per ml for the experiment.

A Sigma® bright-line hemocytometer (Hausser Scientific Company, Horsham, PA) was used to determine if the number of spores per ml that was given on the packaging was correct. A dilution of 4.4 x 10^6 spores per ml was used for the hemocytometer counts. The dilution of spores per ml was based from the label of the Mycotrol® WPO. A method developed by Cantwell (1970) was followed to count spores using the hemocytometer. The central square of the hemocytometer is ruled into 25 squares that are broken down into 16 smaller squares. Total number of spores were counted from five squares, each having 16 smaller squares **(Fig. 4A)**. Spores that were on the top and left lines of the small squares were counted. Any spores that were on the line at the bottom or right side of the square were not counted. It was found that the solution had 2.75 x 10^6 spores per ml which is less than the initial concentration we started out with (4.4 x 10^6 spores per ml). At the time of each test 0.5 ml of concentration C (2.2 x 10^6 spores) was sprayed on an SDA agar plate to determine germination percentage **(Fig. 4B)**. Plates were held for 24 hours and the number of spores that germinated and did not germinate were counted under a compound microscope on four fields of view of the agar plate at 400X magnification. Germination percentages were found to be 66%, 75%, and 40% for the first, second, and third experiment.

Direct sprays

Four known concentrations were used to directly spray adult SWD. A 0.5 ml deionized water control and 0.5 ml of fungal solutions (high=2.2 x 10^8 spores, medium=2.2 x 10^7 spores, and low=2.2 x 10^6 spores per 0.5ml) were applied to the flies.

Flies were anesthetized using FlyNap® (Carolina Biological Supply Co., Burlington, NC) for three minutes and were inactive for approximately 50 minutes **(Fig. 5 A-B)**. This allowed them to be sorted into petri dishes before they were sprayed **(Fig. 6A)**. Ten flies between 1-4 days old were used for each replication. Each treatment and control were replicated 12 times with four replications per treatment dates. Experiments were done on 5 February, 19 Feb and 11 March 2018. On 19 February and 11 March, there were not enough flies to finish the experiment and several replications were done the following day. The 0.5 ml solutions were pipetted into 10 ml spray bottles (Elfenstal) **(Fig. 6B)**. Adult SWD were sorted into petri dishes and then directly sprayed. Filter paper was placed in the petri dishes to help absorb the solutions, so the flies would not drown **(Fig. 6C)**. Once the flies were sprayed, the flies and filter paper were placed in Fabri-Kal® (Kalamazoo, MI) 473 ml polypropylene deli containers with lids. The lids had 5cm x 5cm square cut in the top with a fine mesh glued on them to help with air flow **(Fig. 7)**. Food was placed into each container. After seven days, the number of dead and living flies were recorded.

Fungal sporulation

Seven days after exposure, all dead flies were placed into separate petri dishes by treatment and replicate, containing moist filter paper to determine if the deaths were from *B. bassiana* **(Fig. 8 A-B)**. Once flies were placed into a petri dish, Parafilm® (Bemis®, Neenah, WI) was wrapped around the edges and the dishes were covered by aluminum foil to prevent light. Additional water was added if the filter paper dried out. One week after exposure the number of flies that sporulated was recorded.

Statistical Methods

To test differences for fly death among the treatments an ANOVA was conducted. A Fischer's exact test was conducted to see if there was any difference between sporulation among the treatments. All statistics were done in JMP Pro 13.

Results

No significant differences in percentages of mortality or sporulation were found between the controls and the three fungal treatments ($F_{3,44}=0.8$; $P>0.4953$; Fischer's exact test: $p>0.24$) **(Table 1)**. This could have played a role in the low infection rates of tested flies. Out of the total number flies that died per treatment, very few sporulated. The high treatment had the highest sporulation rate at 1.68% with two flies sporulating (n=119), followed by low treatment at 0.85% with one fly sporulating (n=117) **(Fig. 9)**. Medium treatment had 0% sporulation (n=120) **(Fig. 9)**.

Discussion

In this study the GHA strain of *B. bassiana* was directly sprayed on adult SWD at a higher concentration of 2.2×10^8 spores per 0.5ml than the 1×10^7 spores per ml applied by Woltz et al. (2014) that resulted in sporulation of *B. bassiana* in <2% and < 1% of treated flies, respectively. In comparison, Cuthbertson et al. (2014) caused 44% mortality of adult SWD 7 days after applying the *B. bassiana* strain ATCC 74040 as a 0.3% suspension in Naturalis® (Intrachem Bio International, Geneva, Switzerland). The label for Naturalis® states it is a suspension of conidiospores in vegetable oil, which improves spore germination and UV protection, enhancing the efficacy in the field. Mycotrol® WPO does contain petroleum distillates. Although Gargani et al. (2013) found that *B. bassiana* strain GHA (Botanigard®) and *B. bassiana* strain ATTC74040 (Naturalis®) caused 84% and 54% mortality of SWD, respectively, that experiment did not directly spray the adults. Gargani et al. (2013) immersed infested fruits into the different products to see if it would prevent SWD emergence. This may be why there are different findings.

Fungal germination and hyphal growth are affected by environmental factors such as temperature, relative humidity, and solar radiation (Lacey 2017). Several germination sprays were conducted before the experiments were done. During those times, germination percentages were above 90%. However, when the experiments were conducted the germination rates dropped between 40 to 75%. This may have affected the percentage mortality of treated adult SWD. These treated adult SWD females laid eggs and larvae developed, which was similar to what Cuthbertson et al. (2014) observed.

Entomopathogenic fungi attack the host through the cuticle (Lacey, 2017). With so few flies sporulating, the cuticle of SWD may be able to protect the fly from the fungus and keep it from infecting the flies. A 0.1 gram of *B. bassiana* solution was made and only 0.5 ml was sprayed to keep the flies from drowning. Many fungi need a high threshold of conidia necessary to cause an infection (Lacey 2017). By taking the areas of the petri dish (56.7 mm^2) and the approximate area of a fly (4.25 mm^2) we can estimate how many spores were sprayed per fly. In this experiment, there were 164,773 spores sprayed per fly at the highest concentration of 2.2×10^8 spores per 0.5 ml. If only 66% germinated, then approximately 108,740 spores would have been available to infect each fly. Cossentine et al. (2016) found that *B. bassiana* had a dose dependent impact on SWD mortality. By increasing the spray to 1 ml would increase the number of spores sprayed per fly allowing *B. bassiana* to potentially better infect the flies. Adding a sugar mixture to the fungus might help in getting better infection since the flies would use their proboscis to feed on the sugar (Cowles, n.d.). Adding a sugar solution to a *B. bassiana* formulation could infect the flies faster and not just rely on the fungus to contact the insect's cuticle (Cowles, n.d.). Looking at alternative ways to introduce the fungus to the adult flies might help us understand if *B. bassiana* GHA strain (Mycotrol®) can control SWD. There are other strains of *B. bassiana* that should be investigated further that might be more infective to SWD. In addition, other fungal species may have potential and should be tested.

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Appendix

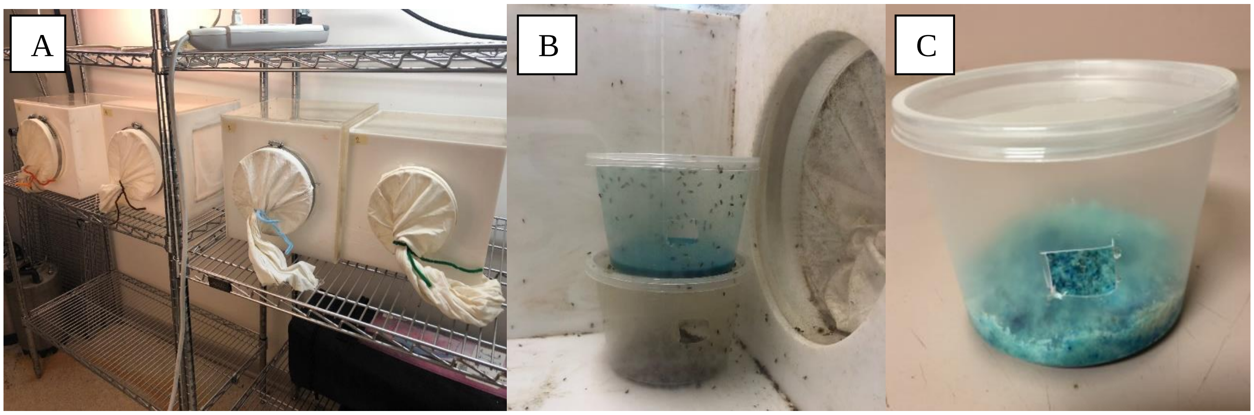

Figure 1: A) Fine mesh cloth screen cages for rearing spotted wing drosophila, B) Deli containers with blue Drosophila media in cages and C) food container with 2 cm hole and lid.

Figure 2: Spotted wing drosophila (SWD) larvae, pupae and flies in vial with Drosophila food.

Figure 3: Commercial formulation of Mycotrol® WPO containing *Beauveria bassiana* strain GHA.

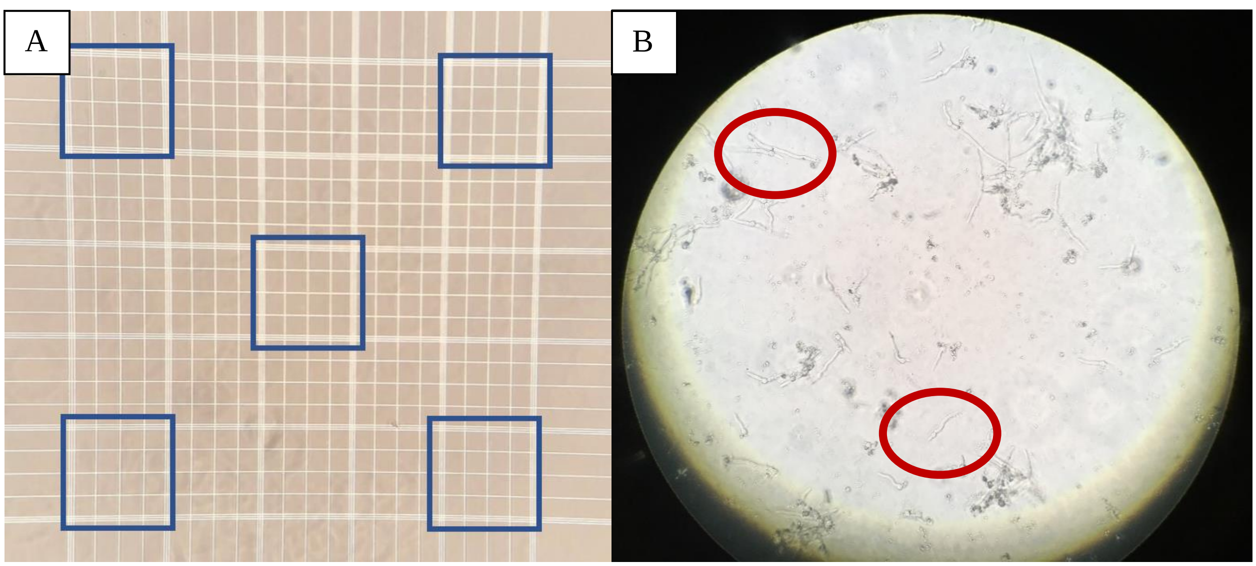

Figure 4: A) *Beauveria bassiana* spores were counted only in squares highlighted in blue on hemocytometer plate and B) low concentration of $2.2x10^6$ germination counts at 400X with germinating spores circled in red.

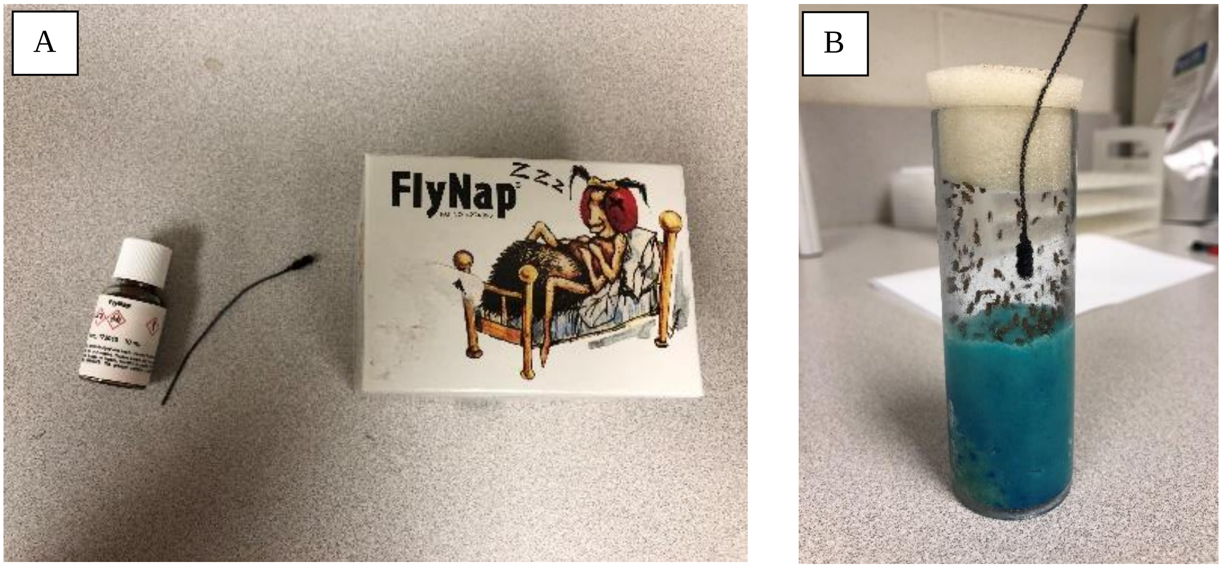

Figure 5: A) FlyNap® anesthetic supplies and B) anesthetic wand dispensing anesthetic into vial to knock down spotted wing drosophila flies.

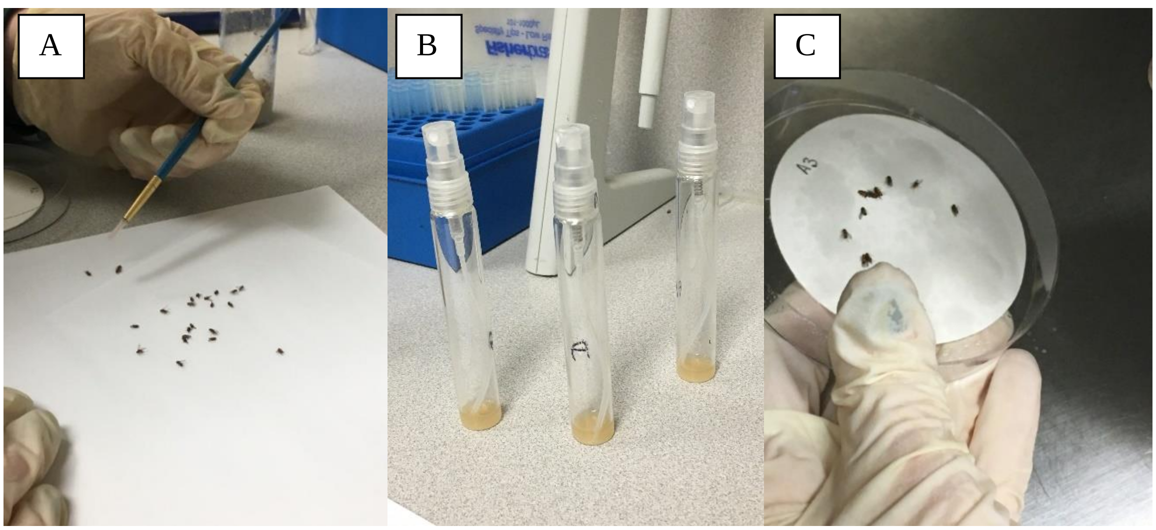

Figure 6: A) Sorting spotted wing drosophila (SWD) flies after anesthetizing. B) Spray bottles used to apply *B. bassiana* to SWD flies. C) Petri dish with filter paper and treated SWD flies.

Figure 7: Deli containers with mesh top used to hold spotted wing drosophila flies for seven days after treatment.

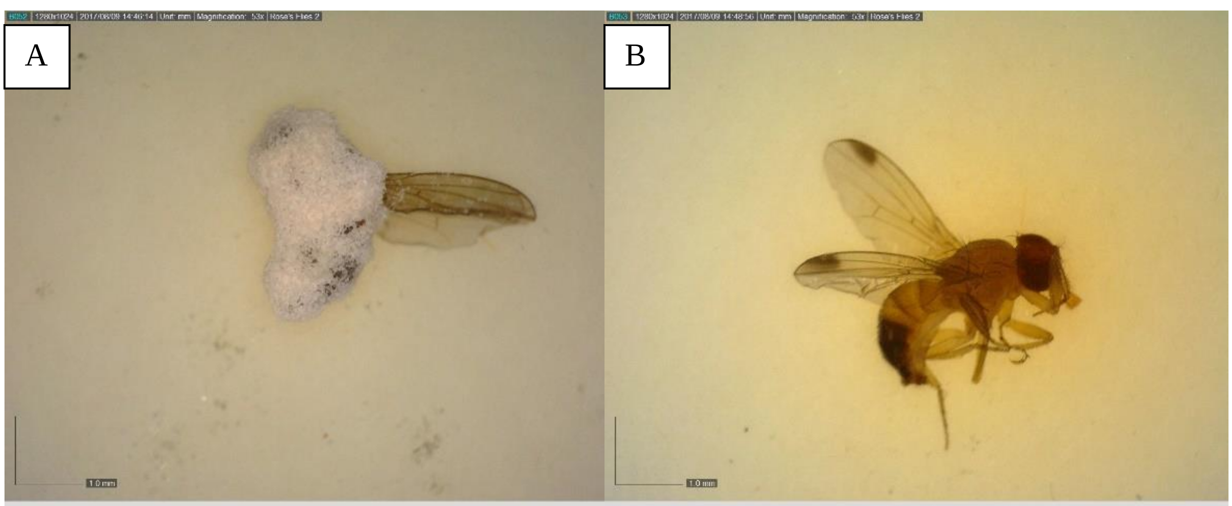

Figure 8: A) *Beauveria bassiana* infected spotted wing drosophila (SWD) fly and B) non-infected SWD.

Table 1: Treatment mean (± SE) percentage mortality and percentage sporulation of *Beauveria bassiana* of spotted wing drosophila (SWD) flies. No significant differences were found between treatments.

Treatment	% mortality after 1 week	% sporulation *B. bassiana*
High = 2.2 x 10^8 spores	1.4 ± 0.7	0.2 ± 0.1
Medium = 2.2 x 10^7 spores	2.4 ± 0.5	0.0 ± 0.0
Low = 2.2 x 10^6 spores	2.2 ± 0.7	0.08 ± 0.08
Water control	1.3 ± 0.6	0.0 ± 0.0

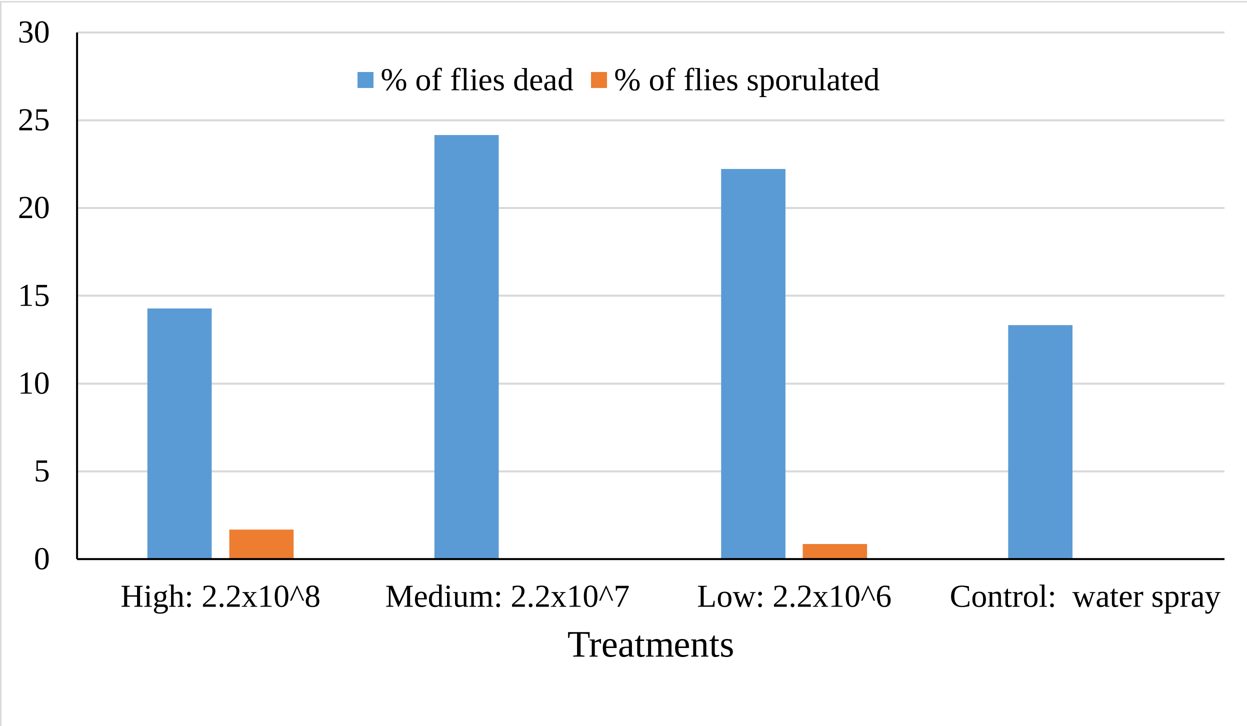

Figure 9: Percentage of spotted wing drosophila (SWD) flies that died and sporulated from being directly sprayed with one of three concentrations of *B. bassiana* strain GHA (Mycotrol WPO) or water control.

Chapter 5: Conclusions

Spotted wing drosophila (SWD) is an invasive fruit fly pest that has spread all over the United States, Canada and Europe (Hauser 2011). Female SWD have a serrated ovipositor that allows them to attack ripening or ripe fruit (Walsh et al. 2011). The objectives of this study were to determine if winter morphs (WM) of SWD were present in Arkansas, how long SWD was active into the winter months, what alternative food sources SWD utilized in Arkansas, and what dose of Mycotrol® (*Beauveria bassiana* strain GHA) caused mortality of SWD flies. The following findings will hopefully help growers better manage this pest in Arkansas.

The winter morphs (WM) are characterized as being larger and darker than summer morphs (SM) (Wallingford and Loeb 2016). It was found in both Arkansas and Tennessee that female SWD caught in the fall (October-December) were darker and larger than those caught in the summer (June-July). These are similar findings to what Wallingford and Loeb (2016) found in New York. In Arkansas, flies caught in May were found not to be smaller or lighter than flies caught in October. Since it is believed that adult SWD overwinter (Kanzawa, 1939; Walsh et al. 2011), the first flies caught could be the ones that survived the winter. Wing length alone may not be a good indicator of a WM. In Arkansas, flies caught in July had no difference in wing length of flies caught in October. However, when looking at pigmentation, there was a difference. With this information, we can better understand the characteristics of a winter morph and why SWD may be able to survive the winter months in areas that it was first thought they could not survive.

Winter activity of SWD was determined by monitoring fly catch in two SWD baited traps from October 2017 to May 2018 near alternative food resources in two locations in Fayetteville, AR. During the winter of 2017-2018, SWD flies were caught in high numbers (average 40 flies caught per trap) into December. It wasn't until the end of December that SWD fly counts declined. In January, only three female SWD were caught. Thereafter, no SWD flies were caught from February to May. Food sources available to SWD from September to November were pokeweed (*Phytolacca americana* L.), trumpet honeysuckle (*Lonicera sempervirens* L.), Carolina buckthorn (*Rhamnus caroliniana* (Walter) A. Gray), bush honeysuckle (*Lonicera maackii* (Rupr.) Herder), and beauty berry (purple) (*Callicarpa americana* L.). Beauty berry was the only one that SWD adults did not emerge from infested fruit. This could be due to cold exposure in field or the laboratory conditions once the fruit were brought in. However, it does tell me that female SWD were still laying eggs into November. In the spring, the only fruiting plant in May that supported SWD was Mulberry (*Morus rubra* L.). These alternative hosts were similar to those reported by Lee et al. (2015) and Herrera (2017). It was found that growers with wooded areas containing alternative resources for SWD captured SWD flies earlier in the spring than growers with little to no wooded areas around their fields (Pelton et al. 2016). Growers with alternative hosts adjacent to their fields might sustain SWD through the winter and expect SWD infestation as soon as their berry crops ripen.

Beauveria bassiana strain GHA (Mycotrol WPO) applied as a contact spray did not kill more than 2% of treated SWD. Only the high (2.2×10^8 spores per ml) and low (2.2×10^6 spores per ml) concentrations of *B. bassiana* sprayed on SWD flies caused infections of 1.7% and 0.9% SWD flies, respectively. The medium concentration caused no fly infection. These findings were similar to that of Woltz et al. (2014). In contrast, Cuthbertson et al. (2014) sprayed a 0.3% suspension of *B. bassiana* strain ATCC 74040 (Naturalis®) that caused 44% SWD fly mortality within seven days of application. Neither *B. bassiana* strain caused enough SWD fly mortality to provide adequate control of SWD. Other strains and formulations of *B. bassiana* that increase ingestion by flies and prolong fungal longevity in field by protecting spores from UV radiation should be developed and evaluated against SWD flies.

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