Diapause Biology, Dispersal Capabilities and Insecticide Use for Lygus Lineolaris in Mid-Atlantic Cotton Systems

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Scientific Abstract

Cotton (*Gossypium hirsutum L.*), is cultivated in the United States, primarily in regions characterized by long, hot summers to optimize plant growth. Virginia is the northernmost state where cotton is grown, with approximately 84,000 acres annually. The unique challenges of cultivating cotton in Virginia stems from its relatively short season due to its geographical location, lack of large contiguous acreage, and distinctive issues with pests. A significant pest of this region is the tarnished plant bug, *Lygus lineolaris* (Palisot de Beauvois), which emerged as a major threat to mid-Atlantic cotton during the late 2010s. *L. lineolaris* utilize a variety of wild and cultivated hosts to survive the winter months. The overwintering success and distribution of diapause survival *L. lineolaris* was measured on cover crops and weeds common in the Mid-Atlantic. Densities varied between weed and cultivated hosts, with *L. lineolaris* exhibiting increased survival in legumes compared to grains. Carbohydrate, lipid and protein levels were measured within diapausing and non-diapausing *L. lineolaris* specimens. Overwintering specimens usually had elevated level of carbohydrates and lipids, while containing decreased concentrations of protein. Nutrient quantification provided an effective tool in selecting for diapause status in *L. lineolaris*. Through the results from this study, an alternative method to dissection for determining diapause status in *L. lineolaris* has been identified. In the spring, movement of *L. lineolaris* throughout the landscape is highly dependent on host senesce. Flight analysis, behavioral assays and nutritional quantification assays on *L. lineolaris* populations from

different weed hosts were performed to assess the flight capacity of specimens fed from different hosts. While weed hosts type provided populations with differing internal nutrient levels, sustained flight was not different between populations. When dispersal of *L. lineolaris* into cotton occurs, insecticide treatments following scouting are often necessary to prevent economic damage to the plant. Insecticide experiments were conducted aiming to assess the impact of different active ingredients on *L. lineolaris*, secondary pests, and natural enemy populations. Findings indicated that insecticides used to control *L. lineolaris* were successful at lowering pest populations and acephate was found to impact natural enemy populations. Plots applied with acephate experienced secondary pest outbreaks, highlighting the crucial role of natural enemies.

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General Audience Abstract

Cotton is cultivated throughout the southern United States, extending up the east coast and into North Carolina and Virginia. Virginia poses unique challenges for cotton growers due to specific pest issues and need for region-specific research. The tarnished plant bug is a key pest of mid-summer cotton across this region. Overwintering studies were conducted to investigate host preferences on cover crop and weed hosts, as well as diapause survival and termination timing on cover crops. These studies revealed that hairy vetch and deadnettle species may harbor larger populations compared to other weed hosts, and that cover crops such as hairy vetch and crimson clove led to greater overwintering survival compared to grains. During diapause, metabolic changes occur that can lead to increased fat body to aid in overwintering survival. Diapausing and non-diapausing tarnished plant bugs were used in assays to quantify differences in the nutrients commonly found in this fat body. This study successfully found differences in carbohydrates, lipids and proteins levels between the diapausing and non-diapausing populations. This may allow for the identification of reproductive status of tarnished plant bugs based on nutrient levels. Flight capacity, activity levels and nutrient levels in tarnished plant bug populations taken from spring and summer weed hosts were investigated, with the objective to understand the ability of these populations to transition to cotton in the mid-summer. There were some indicators that flight initiation may be influenced by weed host, while all populations had similar flight potentials. Once in cotton, tarnished plant bugs are primarily treated with chemical

insecticides which may produce unknown consequences to the system as a whole. Experiments were conducted to assess the impact of insecticides on non-target species within cotton were conducted in the field. Certain insecticides were identified to be harmful to non-target and potentially beneficial insect species, which contributed to outbreaks of other secondary pest species.

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Challenges in mid-Atlantic cotton in relationship with *Lygus lineolaris* as a pest

The tarnished plant bug, *Lygus lineolaris* (Palisot de Beauvois) (Hemiptera: Miridae) is a major pest of cotton (*Gossypium hirsutum*) in the Mid-Atlantic. Historically, *L. lineolaris* was a minor pest of cotton, but has become better established cotton, emerging as a key pest throughout the United States, beginning in the mid-South, and in the Mid-Atlantic by the late 2010's (Williams, 2007; Williams, 2017). Control for this pest relies on insecticide applications and the potential development of resistance in *L. lineolaris* populations is a concern. Understanding this insect's ecology and physiology, including diapause and flight, can inform the implementation of integrated pest management (IPM) strategies in cotton across the region.

During the winter, *L. lineolaris* survives adverse conditions (i.e. low temperatures) by entering a state of diapause, which results in metabolic and physiological changes. Diapausing insects often increase fat body to store nutrients during food scarcity and prevent the damage to tissues as a result of ice crystal formation (Lees, 1955). In *L. lineolaris*, fat bodies and underdeveloped reproductive organs are used to identify individuals in diapause (Snodgrass, 2003). Termination of diapause is dependent on conditions such as temperature, photoperiod, and nutrition status; and ends when the insect becomes reproductive (Snodgrass et al., 2012). Lack of nutritious food sources over the winter could delay diapause termination and, subsequently, the appearance of the first generation of nymphs in the spring.

L. lineolaris feed on the meristematic tissue of various weedy hosts from spring to early summer (Young, 1986; Esquivel & Mowery, 2007). By the mid-summer, weed hosts present

around fields senesce, and *L. lineolaris* move from weed hosts and into cotton and other cultivated hosts (Outward et al., 2008). There is limited knowledge about the physiological mechanisms triggering this movement across the landscape, and the role of weed hosts supporting the dispersal of this insect into cotton. Information on how weed hosts support diapausing populations and influence dispersal capacity can be included on proposing weed management around cotton fields as a potential IPM strategy.

Once *L. lineolaris* adults disperse into cotton and rise above threshold, chemical controls are suggested for *Lygus* (Musser et al., 2009; Aghaee et al., 2019). Insecticide applications, including active ingredient selection and timing, can be optimized to support IPM strategies and to preserve natural enemies that contribute to pest control. Predatory arthropods, like ladybird beetles (Coleoptera: Coccinellidae), lacewings (Neuroptera: Chrysopidae), big-eyed bugs (Hemiptera: Geocoridae), minute pirate bugs (Hemiptera: Anthocoridae), assassin bugs (Hemiptera: Reduviidae), and spiders (Araneae) are present within cotton systems. Several active ingredients sprayed in cotton can result in high mortality of these predatory arthropods. This reduction of beneficials can lead to the outbreak of secondary pests in treated fields.

In my dissertation, I conducted research to provide information on *L. lineolaris* physiology, biology and control. The objectives for this dissertation were to (1) evaluate overwintering success and host preferences of *L. lineolaris* on cover crops and weed hosts; (2) quantify nutritional differences between diapausing and reproductive *L. lineolaris*; (3) assess differences in flight potential, activity, and nutrient levels between *L. lineolaris* fed on common weed hosts; and (4) evaluate the impact of insecticides used to control *L. lineolaris*, other pests and natural enemy populations.

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Chapter 2

Lygus lineolaris (Hemiptera: Miridae) diapause survival and termination on winter cover crops

Abstract

The tarnished plant bug, *Lygus lineolaris* (Palisot de Beavois), overwinters in diapause as an adult. They terminate diapause more readily when nutritional hosts are available, opposed to when using poor hosts or dead plant debris. Adult populations were collected from weed hosts during the fall of 2022 and 2023. These populations were placed in outdoor cages containing cover crops such as winter wheat (*Triticum aestivum*), rye (*Secale cereal*), crimson clover (*Trifolium incarnatum*), and hairy vetch (*Vicia villosa*). *L. lineolaris* adults were taken from cages and had their diapause status assessed to determine if reproductive initiation was influenced by host type. Adults collected from hairy vetch terminated diapause faster compared to those on winter wheat. Survival within cages were highest in hairy vetch, and crimson clover were higher compared to *L. lineolaris* overwintered on wheat and rye. In addition, some in-field choice assays were performed using the cage approach. The host plants studied for these choice assays included the previously mentioned cover crops, fescue (*Festuca spp.*), curly dock (*Rumes crispus*), purple deadnettle (*Lamium purpureum*), and henbit deadnettle (*Lamium amplexicaule*). Higher *L. lineolaris* densities were observed on hairy vetch and deadnettle, when compared to fescue, and curly dock. The implications of these findings may influence winter weed control and cover crop choice to impact populations of *L. lineolaris* capable of infesting cotton later in the year.

Key words: overwinter, distribution, survival, weeds, cotton

Introduction

The tarnished plant bug, *Lygus lineolaris* (Palisot de Beauvois) (Hemiptera: Miridae) is a polyphagous insect widely distributed throughout the United States. *L. lineolaris* has been documented feeding over 350 plant species across North America, while standing as a notable economic pest in diverse agricultural systems (Young, 1986). In the mid-Atlantic, *L. lineolaris* poses a substantial economic threat to cotton (*Gossypium hirsutum*), through its feeding activity on meristematic tissue, resulting in the abscission of squares and bolls causing a loss of harvestable cotton lint (Layton, 2000, Williams 2017). Control strategies heavily rely on frequent applications of insecticides, a practice that has led to resistance for specific insecticide classes, such as pyrethroids and organophosphates (Parys et al., 2017; Dorman, Gross, et al., 2020). Given the pressing need for alternative control methods in cotton systems, exploring *L. lineolaris* biology may offer additional information for improving its management.

L. lineolaris has the ability to enter diapause, a physiological alteration to endure cold temperatures and food scarcity during winter (Lees, 1955; Bariola, 1969). As days shorten in the fall, nymphs are induced to enter into diapause upon reaching adulthood. The critical photoperiod for *L. lineolaris* where 50% of the unhatched eggs are induced into diapause is measured at 12.5:11.5 h Light:Dark (Bariola, 1969; Tauber et al., 1986; Villavaso and Snodgrass, 2004). Diapause initiation triggers metabolic changes within the insect, leading to the slowing of the metabolism, reduced locomotion activity, and an increase in hypertrophic fat body (Snodgrass, 2003; Brent et al., 2013). Unlike similar *Lygus spp.* such as *Lygus hesperus*, *L. lineolaris* does not undergo any discernible external phenotypic changes indicating their

diapausing status (Snodgrass, 2003; Brent et al., 2013). Instead, diapausing status of *L. lineolaris* can be identified through close examination of the actual fat bodies and the status of reproductive organs (Lees, 1955; Snodgrass, 2003).

The termination of diapause in *L. lineolaris* is influenced by various factors and stimuli (Tauber et al., 1986; Snodgrass et al., 2012). *Lygus lineolaris* sustain diapause when exposed to photoperiods of 10:14 h (L:D) or less (Villavaso and Snodgrass, 2004) . The temperature of 10°C is identified as the minimum necessary temperature for the development of *L. lineolaris* eggs and nymphs, emphasizing its potential role in *L. lineolaris* diapause regulation (Ridgway and Gyrisco, 1960; Fleischer and Gaylor, 1988; Villavaso and Snodgrass 2005). This temperature is also considered to be the temperature point that allows *L. lineolaris* to terminate diapause (Villavaso and Snodgrass 2004). In order for diapause termination to occur, populations need exposure to both a photoperiod of 10:14 (L:D) and temperatures exceeding 10°C, however, the availability of nutritious food sources may replace one of these conditions, allowing differences in termination rate among local populations (Snodgrass et al., 2012). In all termination situations, male *L. lineolaris* are more likely to break diapause faster than females from stimuli such as food and temperature (Snodgrass, 2003; Snodgrass et al., 2012).

During diapause, *L. lineolaris* can be found on dead plant debris and on various living weed hosts (Kelton, 1975; Snodgrass et al., 1984). In the late fall, adults and nymphs are commonly found on ragweeds (*Ambrosia spp*.), goldenrod (*Solidago spp.*), and frost aster (*Aster pilosus*), the quality of which may enhance the ability of nymphs to enter diapause. As these plants senesce, *L. lineolaris* move to species such as deadnettles (*Lamium spp.*) and curly dock (*Rumes crispus L.*), in the winter months (Cleveland, 1982; Snodgrass et al., 1984; Snodgrass et al., 2004). Simultaneously, cover crops could offer large number of acres of living plant material

available to *L. lineolaris* as potential harboring sites. In the mid-Atlantic, winter wheat (*Triticum aestivum*), and rye (*Secale cereale*) are common cover crops, while crimson clover (*Trifolium incarnatum*), and hairy vetch (*Vicia villosa*) have been planted at an increased rate (Fan et al., 2020; Denton et al., 2023).

The objective of this study was to investigate the influence of plant hosts on the diapause status of *L. lineolaris*. Winter cover crops and cold-season weeds, serving as potential nutritional sources and harboring sites, may play a pivotal role in shaping the survival of overwintering *L. lineolaris*. Moreover, the nutritional options provided by these cover crops could potentially expedite the termination of diapause for *L. lineolaris*, thereby impacting the population dynamics of this pest during the dispersion to cotton in mid-summer. This research also aimed to document the relationship between plant hosts and the diapause biology of *L. lineolaris*, contributing valuable insights into population dynamics of spring populations.

Materials and Methods

1.1. Overwintering on Cover Crops

Selected cover crop plants were grown from seeds in 61 x 61 x 91.4 cm mesh cages during 2021 and 2022, that were expanded to 71.1 x 71.1 x 121.9 cm in 2023. The bottom 15-20 cm was buried into the ground and filed with Miracle-Gro Garden Soil All Purpose (Scotts Miracle-Gro Company, Marysville OH) with additional nutrition supplemented with Osmocote Flower and Vegetable (Scotts Miracle-Gro Company, Marysville OH) at a rate of 45.36 g per cage. A set of cages were planted with a single cover crop plant variety (experimental treatments) of either 1) winter wheat, 2) cereal rye, 3) crimson clover, and 4) hairy vetch. Winter wheat was selected as a

control after *L. lineolaris* overwintered on plant debris experienced 100% mortality. Cages were set up as a completely randomized design, with collection year as the replicate. Cover crops were planted on 22 September 2021, 5 October 2022, and 27 September 2023. Sites for this study were Prices Fork Research Center in Blacksburg VA during 2021, and the Tidewater Agricultural Research and Extension Center (TAREC) in Suffolk VA during 2022 and 2023.

L. lineolaris adults were collected from fields during the fall of each year from weed hosts from the *Asteraceae* family including golden crownbeard (*Verbesina encelioides*), ragweed (*Ambrosia spp.*), goldenrod (*Solidago spp.*), and dog fennel (*Eupatorium capillifolium*) using sweep nets. In 2021, adults were collected from Kentland Farm in Blacksburg VA, and in 2022 and 2023, adults were collected from Whitmore Farm in Wakefield VA, and from the TAREC in Suffolk VA. Adults were put in 30.5 x 30.5 x 30.5 cm mesh cages and feed green beans and water while the experimental cages with cover crops and weeds were growing. Collected *L. lineolaris* were added to cages on 23 November 2021, 30 November 2022 and 29 November 2023, with 220 adults added to each cage in 2021, and 250 adults added in the following years. Beginning the first week of February, 20 adults were taken weekly for four weeks from each cage and immediately euthanized in a -20°C freezer. Adults were dissected following the protocol from Snodgrass (2003) to determine diapause status of each specimen. Adults were sexed through presence of ovipositor and the identification of internal reproductive organs. Diapause status was identified through the existence of hypertrophic fat bodies around the reproductive organs and were determined to be reproductive if 1) the fat bodies were reduced and the reproductive organs were enlarged or 2) if the presence of eggs or white fluid in the accessory glands or seminal vesicles could be identification. Total adults recovered were counted and compared against the total number of adults placed in the respective cage, once these

experiments were terminated. Weather data for all years was collected from the Virginia Tech weather stem program (https://vt-arec.weatherstem.com/).

1.2. Plant Host Distribution

These experiments were performed in 2022 through 2023, following the protocols for establishing cages described in methods section 1.1. Cover crop cages were planted with 1) winter wheat, 2) cereal rye, 3) crimson clover, and 4) hairy vetch, and weed host cages were planted with 1) fescue (*Festuca spp.*), 2) hairy vetch, 3) curly dock, and 4) a combination of purple deadnettle (*Lamium purpureum L.*) and henbit deadnettle (*Lamium amplexicaule*). Hairy vetch was included twice as it is considered both a cover crop and a weed and could be further used to make comparisons between cage types. These plants were each given a quarter of the cage and distribution of the plants was determined randomly with two cages per location of both cover crops and weeds. Cages were set up in a completely randomized design with year and cage as the replicate. Crops were planted on 5 October 2022, and 27 September 2023 at TAREC in Suffolk VA in both years and Wakefield VA in 2023. Percent coverage of weed hosts varied, roughly ranging between 30-70% for fescue, 10-20% for curly dock, 40-60% for purple deadnettle and henbit deadnettle, and 80-90% for hairy vetch. Corrugated plastic dividers were added to each cage that went wall to wall and 71cm in height which separated the plants within the cages. A subpopulation of the *L. lineolaris* collected for the overwintering experiment were placed in each cage with 150 adults added per cage in 2022, and 100 adults added in 2023. Differences in adults added between years were due to an increase in cages, and high mortality of adults before infestation in 2023. *L. lineolaris* were added on 30 November 2022 and 29 November 2023, with sampling beginning the first week of December, and continuing once per

week through the last week of January. Sampling was performed in each plant quadrant within the cage with the total number of *L. lineolaris* counted over the period of one minute.

1.3 Data analysis

Data was analyzed using R (Version 4.x3.1 R Core Team, 2023) through the 'lme4' (Bates et. al., 2015), 'emmeans' (Lenth, 2018), 'car' (Fox et. al., 2019) and 'multicomp' (Hothorn et. al, 2023) packages. *L. lineolaris* overwintering on cover crops were analyzed for diapause status and recovery. Status of adults in diapause versus reproductive were analyzed by sex using a binomial distributed generalized linear mixed model (GLMM), with cover crop and sampling week along with their interaction as fixed effects and year as the random effect, which accounted for repeated measures. Overwintering adult recovery of *L. lineolaris* on cover crops was analyzed using a binomial distributed GLMM with cover crop as the fixed effect and year as the random effect. Data on adult distribution among plant hosts was analyzed using a Poisson distributed GLMM for cages containing weeds and cages containing cover crop. Fixed effects were plant type, sampling week and their interaction, and the combination of collection year and cage ($r = 4$) as random effects, which accounted for repeated measures. All GLMM models were created using the 'lme4' package. Summary statistics of all models were calculated using the 'car' package. Mean separations of all models were calculated using a Tukey's honest significant difference post hoc test with an $\alpha = 0.05$ through the 'emmeans' package. Compact letter assigned to all post-hoc mean separations were displayed executing the 'multicomp' package.

Results

Overwintering on Cover Crops

The first year of the study (2021-2022) in Blacksburg VA, experienced the coldest temperatures in over the three years, with 65 days from December through February reaching temperatures below 0°C and only 41 days exceeding 10°C. The following years of the study, performed in Suffolk VA, cages were exposed to more temperate conditions in 2022-2023 compared to 2023-2024 (**Table 2.1**) All populations were recovered at a level that allowed for modeling from early February through the following four weeks. Populations overwintered on winter wheat and rye persisted until the third week of February 2022, and the fourth week of February 2023 and 2024. Overwintering adults from crimson clover persisted until the first week of March in all years. Adults on hairy vetch in single crop cages remained until the first week of March in the first two years, with all adults taken out of the cages by the end of February in the final year.

Diapause termination of adult male *L. lineolaris* was not influenced by cover crop (χ^2 = 2.86, df = 3, P = 0.4137) or by week sampled (χ^2 = 2.70, df = 3, P = 0.4412). The interaction between cover crop and week did not influence diapause termination in adult males (χ^2 = 7.26, df = 9, P = 0.6105). In females, diapause termination was not influenced by cover crop (χ^2 = 1.76, df = 3, P = 0.6247) or by week (χ^2 = 2.69, df = 3, P = 0.4421), nor the interaction between these two factors (χ^2 = 15.24, df = 9, P = 0.0845).

Lygus lineolaris recovery was influenced by cover crop (χ^2 = 109.40, df = 3, P < 0.0001; **Figure 2.1**). Overall adult recovery across cover crops and years was 31.57%. Adults overwintering on hairy vetch had the highest recovery rate at 44.74%, with crimson clover at 34.99%, followed by wheat at 25.45% and rye at 20.94% (**Table 2.2**). On 16 March 2022 and at the end of first year of the study, a high level of first and second instar nymphs were observed in the hairy vetch cages while no nymphs were observed in cages on any other crop or in any other

year. In 2024, tearing was a common struggle among overwintering cages and required repair throughout the winter with an overall *L. lineolaris* recover rate of 29.80%, which was consistent with previous years. During the winter of 2024, parasitization of *L. lineolaris* by an unknown species occurred at a low level within the cages (data not shown), which may have reduced recovery rate.

Plant Host Distribution

Throughout the study, data collection primarily occurred when temperatures were at or above 10°C with only one instance where data collection occurred below 0°C (2023 at the Wakefield VA site). The interaction between cover crop and week did influence plant host choice (χ^2 = 44.45, df = 21, P = 0.0020; **Figure 2.2.**). Each cover crop maintained over 90% coverage in their respective quadrants in both years. In cages containing weed hosts, the interaction between weed host and sampling week was not significant in affecting the distribution of adults among hosts (χ^2 = 30.31, df = 21, P = 0.0859). Weed hosts did influence the distribution of adults within cages (χ^2 = 24.98, df = 3, P < 0.0001; **Figure 2.3A.**), along with sampling week (χ^2 = 18.89, df = 7, P = 0.0086; **Figure 2.3B.**) in total adults counted. Hairy vetch contained the highest populations of *L. lineolaris,* with an abundance of 7.44 per quadrant, followed by deadnettle spp., with an abundance of 2.92 per quadrant. The number of adults in fescue and curly dock were similar to each other with an abundance of 1.52 and 1.13 per quadrant, respectively (**Table 2.3**). Lower *L. lineolaris* counts occurred in the third week of December.

Discussion

This study aimed to investigate how cover crops and winter weeds may affect the overwintering biology of *L. lineolaris* in Virginia. Adults overwintered in cages on cover crops were dissected to measure diapause termination rates across the month of February, and no differences were found between populations. Recovery rates for adults in cages were used to measure survivability on cover crops. The highest recovery rates were on the cover crops hairy vetch followed by clover, with the lowest recovery rates on winter wheat and rye. An additional cage experiment was added focusing on various cover crops and weed hosts to see if there was a correlation between diapause termination rates and host distribution. Wheat and rye as cover crops were less frequented host plants by adults when compared to cover crops hairy vetch and crimson clover, with results being highly variable among dates. Hairy vetch as a winter weed had higher populations of overwintering adults followed by deadnettle species, as both had greater populations than curly dock and fescue.

Adult recovery from cage studies could be linked to the well-being and survival of *L. lineolaris* on the proposed experimental cover crops. Differences in adult recovery were documented between populations on cultivated grains and legumes. Crimson clover and hairy vetch had higher recovery rates when compared to wheat and rye, with hairy vetch having the highest recovery rates for adults. Although recovery is not a complete representation of *L. lineolaris* survival under field conditions, it could provide some insight into how populations may fare overwinter (Paul et. al., 2024). Cover crops that support populations through the winter may offer higher recovery rates for *L. lineolaris* and the ability to better withstand harsh conditions (Zavalnitskaya et. al., 2022). Nymphs found in hairy vetch cages indicate that this cover crop could be used successfully as an overwintering host and as a reproductive host in the spring allowing elevated populations to be present in both the winter and spring. While available

nutritional hosts during overwintering periods may improve chances of adult recovery and survival, differences in microclimates, such as temperatures within the plant canopy, have been strongly linked to impacting overwintering success through the creation of favorable conditions within certain cover crops (Danks 2012; Snodgrass et. al., 2012).

Comparing population densities in cover crops aimed to explore the potential correlation between overwintering success and *L. lineolaris* overwintering dispersal. Differences between cover crops terminating diapause were not found, as *L. lineolaris* densities within cages varied from one sampling week to another. This variation over time was expected since environmental conditions changed. Nutritional needs could have been met for adults through moving between a host containing low nutrition with a better microhabitat and a more favorable food source (Hahn and Denlinger 2011). Despite not seeing statistical differences in this experiment, *L. lineolaris* have been found in higher densities in fields planted with cover crop mixes containing hairy vetch and crimson clover compared to those that included rye (Tillman et. al., 2004). Rye, crimson clover and hairy vetch have all been found to promote natural enemy abundance during the winter months in future cotton fields (Tillman et. al., 2004; Bowers et. al., 2020).

Studying the relationship between winter weed host plants and the distribution of adults was motivated by the overlapping presence of hairy vetch and clover as both cover crops and weed species. Clover was excluded from the weed host choice cages due to the already observed relationship in cover crop cages between hairy vetch and crimson clover. The addition of purple and henbit deadnettle aimed to establish some parallels with a study involving henbit deadnettle by Snodgrass et al., (2012), which recognized this plant as important hosts of *L. lineolaris* in the mid-South during the winter months. In the mid-Atlantic, deadnettle is an important weed, as it normally appears at high levels in fields prior to cover crop emergence (Yelverton, 2017;

Peacock, 2022). Curly dock struggled to grow in the cages, although sprouted plants did successfully overwinter, potentially due to regional temperature differences between the mid-Atlantic and studies performed in the mid-south, which have considered curly dock as a quality overwintering host for *L lineolaris* (Cleveland 1982, Snodgrass et. al., 1984). Differences in adult distribution among cover crops and weeds may have been influenced by host quality and differences in temperature provided by the microhabitats of the host plant. Microhabitats within weed hosts were expected to differ more than those seen among cover crops, due to the vast differences in foliage and overall growth between weeds included in this study (Danks 2012). A higher number of adults were documented distributing across hairy vetch, while purple and henbit deadnettle harbored a higher population than fescue curly dock. Curly dock was the weed host with the lowest presence of adults, but was potentially impacted by low production of vegetation, as the plant is more prone to develop roots at low temperatures compared to foliage (Maun and Cavers 1970). This may make it a less likely overwintering host in the mid-Atlantic compared to the mid-south (Cleveland 1982; Snodgrass et. al., 1984).

In mid-Atlantic cotton growing regions, strategic consideration of winter cultural controls and cover crop selection holds the potential to create less favorable conditions for diapausing *L. lineolaris*. The increase in *L. lineolaris* populations, coupled with the promotion for alternative winter cover crops (Fan et al., 2020; Denton et al., 2023) such as hairy vetch, could result in elevated pest levels in mid-summer cotton by potentially allowing *L. lineolaris* to overwinter more effectively on those introduced cover crops. Results from this study highlighted the influence of cover crops like winter wheat and rye on the reduced rate of adult recovery, which ultimately could contribute to lesser the overwintering success of *L. lineolaris*. Plants such as hairy vetch and clover, present as cover crops or weeds, may result in higher populations of *L.*

lineolaris in and around future cotton fields due to their ability to promote the presence of adults, while potentially supporting survival rates that allow for even greater spring populations. Other weeds like purple and henbit deadnettle can have similar effects in supporting increased adult population during the winter months compared to other weeds and other cover crops such as wheat and rye. Selection of winter cover crops can incorporate the information from these experiments to influence *L. lineolaris* populations. Additionally, the removal of weeds such as hairy vetch and deadnettles may negatively influence the dynamics and recovery of overwintering adults, thus reducing overall overwintering success and potentially easing pest pressure on summer cotton.

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Table 2.1. Average monthly temperatures (°C ± SE) during *L. lineolaris* overwintering time period for cover crop experiments in this project (temp = Temperature; No. = Number; d = days)

Year	Month	Location	High temp	Low temp	No. d 0°C or less	No. d 10°C or greater
2021	Dec.	Blacksburg	0.03±0.97	14.47±0.81	16	23
2022	Jan.	Blacksburg	-5.57±0.91	05.10±1.01	29	3
2022	Feb.	Blacksburg	-3.09±1.04	10.69±1.02	20	15
2022	Dec.	Suffolk	-0.39±1.12	11.85±0.96	18	20
2023	Jan.	Suffolk	2.79±0.90	14.86±0.84	13	27
2023	Feb.	Suffolk	5.88±1.25	16.85±1.57	3*	17*
2023	Dec.	Suffolk	2.14±1.12	15.10±0.70	16	28
2024	Jan.	Suffolk	0.27±1.11	11.91±1.04	18	18
2024	Feb.	Suffolk	0.56±0.85	13.09±0.66	15	22

*Station weather data was not recorded between 4 – 8 February 2023

Table 2.2. Contrasts for *Lygus lineolaris* recovery on cover crops

Contrast	z ratio	95% CI	P value
Wheat/Rye	0.44	0.59-2.09	0.9708
Wheat/Crimson Clover	-4.40	0.22-0.67	0.0001***
Wheat/Hairy Vetch	-7.89	0.10-0.31	0.0000***
Rye/Crimson Clover	-4.78	0.19-0.61	0.0001***
Rye/Hairy Vetch	-8.20	0.09-0.29	0.0000***
Crimson Clover/Hairy Vetch	-3.89	0.28-0.77	0.0005***

Asterisks represent significant significances (*P<0.05, ***P<0.001)

Table 2.3. Contrasts for *Lygus lineolaris* distribution on weeds

Contrast	z ratio	95% CI	P value
Fescue/Curly Dock	1.24	0.73-2.46	0.5999
Fescue/Deadnettle	-3.49	0.32-0.84	0.0026*
Fescue/Hairy Vetch	-9.85	0.13-0.31	0.0000***
Curly Dock/Deadnettle	-4.39	0.22-0.68	0.0001***
Curly Dock/Hairy Vetch	-9.71	0.09-0.25	0.0000***
Deadnettle/Hairy Vetch	-7.25	0.28-0.55	0.0000***

Asterisks represent significant significances (*P<0.05, ***P<0.001)

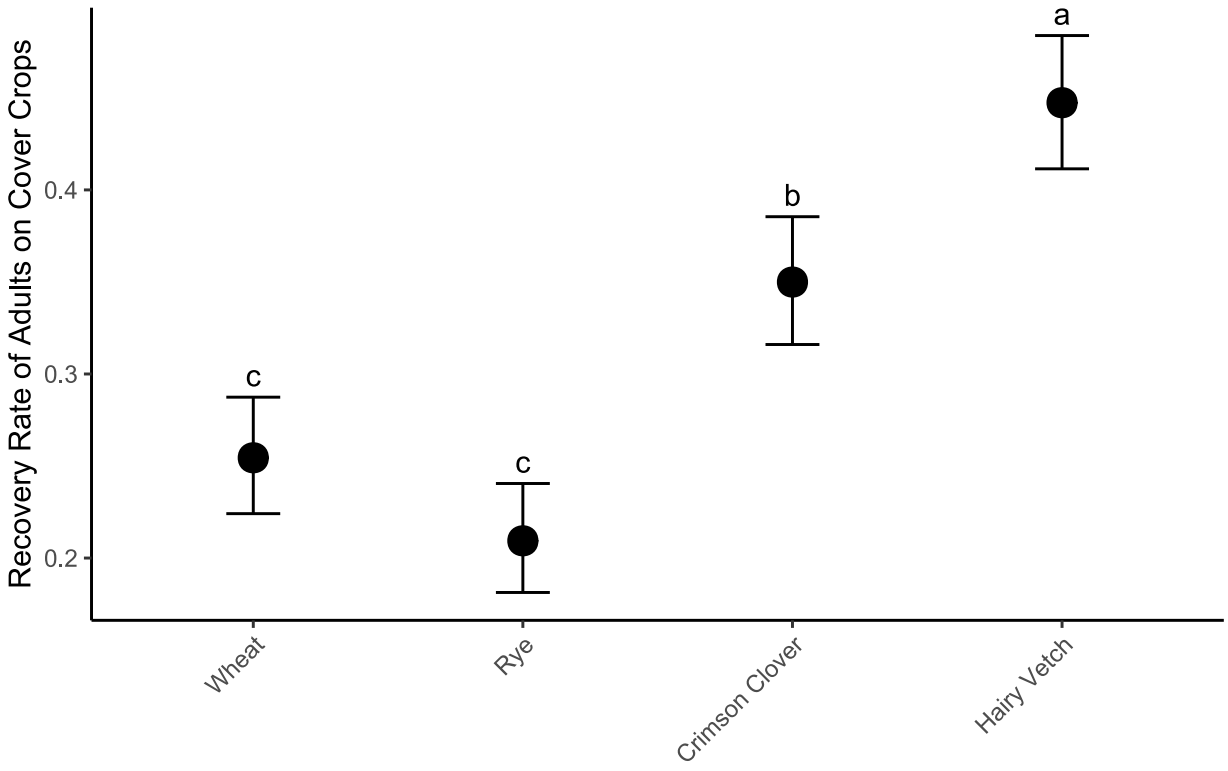

Figure 2.1. Average recovery rates ± 95% Confidence Interval (CI) of *L. lineolaris* adults overwintered in singular cover crop (X-axis) cages across all years of the experiment. Starting populations in cages processed in 2022 were 220 adults per crop, which increased to 250 in 2023 and 2024. Different letters over the means for cover crop rates indicate significant differences as determined by Tukey's HSD test ($\alpha = 0.05$).

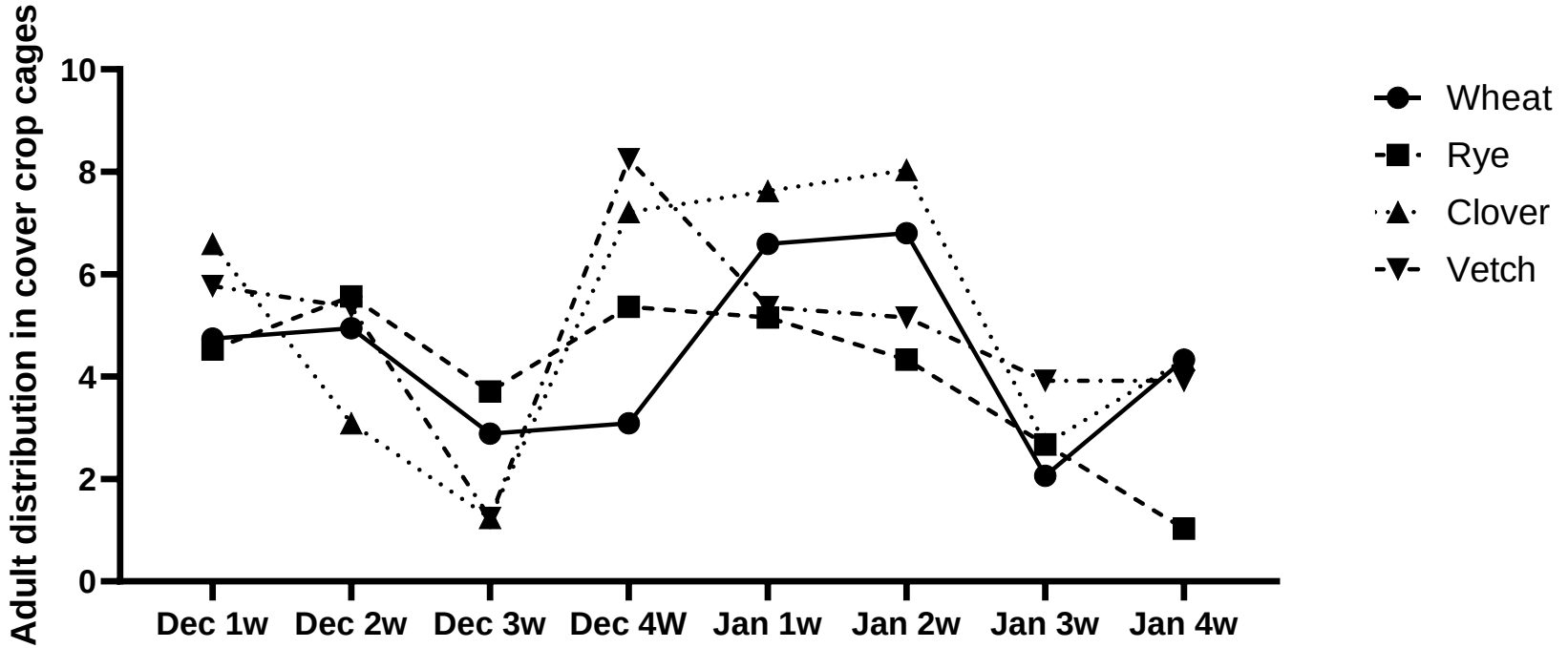

Figure 2.2. Average number of *L. lineolaris* adults on cover crops (different lines) across each sampling week (X-axis). Sampling weeks are expressed as abbreviation of the month (Dec = December) and the week of the month (1w = first week). Lines represent *L. lineolaris* densities on the cover crops wheat, rye, crimson clover and hairy vetch.

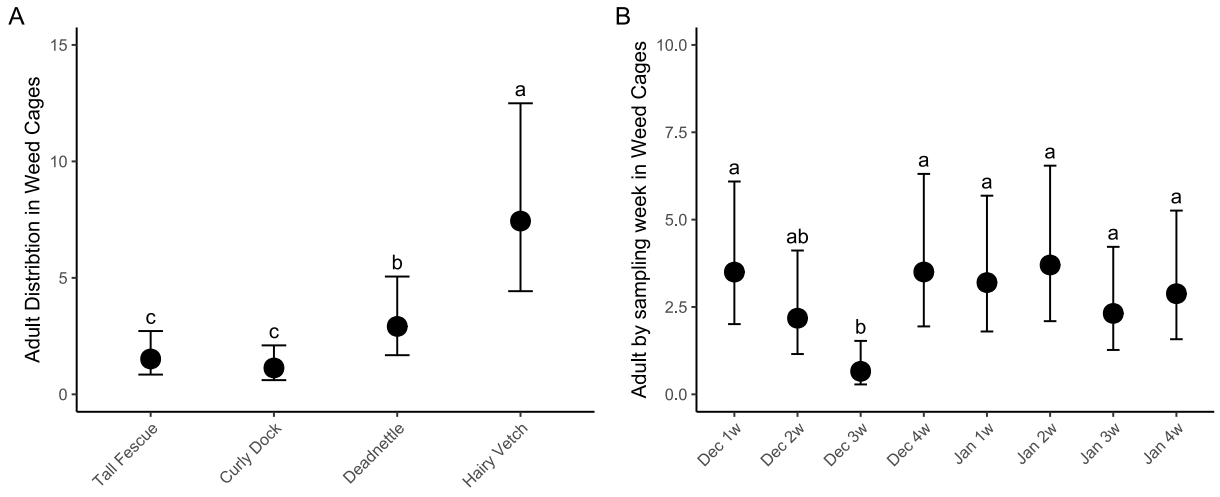

Figure 2.3. Average number of *L. lineolaris* adults on (A) weed hosts and (B) across sampling week. Bars represent the upper and lower 95% confidence intervals. Different letters over bars indicates significant differences among weed plants, or across sampling week, as determined by Tukey's HSD test ($\alpha = 0.05$).

Chapter 3

Protein, carbohydrate, and lipid presence in diapausing *Lygus lineolaris* (Hemiptera: Miridae) populations

Abstract: Diapause in adult tarnished plant bugs, *Lygus lineolaris* (Palisot de Beauvois) (Hemiptera: Miridae), induces changes in stored nutrients compared to reproductive individuals. Hypertrophic fat bodies, a key characteristic of diapausing individuals, are primarily composed of proteins, carbohydrates, and lipids, and serve to enhance winter survivability in adverse conditions. The presence of these fat bodies could play a crucial role in determining reproductive status in *Lygus* species. Quantification assays were conducted on *L. lineolaris* with known reproductive status to assess whole-body nutrient levels. Diapausing *L. lineolaris* exhibited higher carbohydrate and lipid levels, along with lower protein levels, compared to their reproductive counterparts. Moreover, the differences in protein and lipid levels between the two populations (overwintering vs. reproductive) were distinct enough to potentially determine reproductive status through these respective and proposed assays.

Keywords: nutrient quantification, fat body, overwinter, physiology, plant bug

1. Introduction

The tarnished plant bug, *Lygus lineolaris* (Palisot de Beauavois), is a pest of several North American crops including spring nursery plants, fruits, seeds, and cotton (Tingey and Pillemer 1977; Young 1986). The range of this agricultural pest extends from Mexico, through the United States, and into Canada. In these different regions, the tarnished plant bug can have two to six generations per year (Knight 1941; Capinera 2001). During the final generation, the plant bug responds to a shorter photoperiod (Bariola 1969; Villavaso and Snodgrass 2004), leading to diapause and allowing the adult *L. lineolaris* to survive winter conditions. As a result of diapause, there is a suppression of feeding behavior, allowing the plant bug to prepare for extended periods of potential food scarcity. Further, physiological changes modify the function of the ovaries and accessory glands, which conserves resources and prevents reproduction, but also results in hypertrophic fat body around insect reproductive organ, providing an energy reserve that enhances survival during times of food scarcity (Lees 1955, Bariola 1969). Termination of diapause is determined by favorable temperatures, longer photoperiod and the presence of nutritious food sources, the combination of which drives *L. lineolaris* to enter a reproductive state (Snodgrass et al., 2012).

The state of the fat bodies is vital in the process of diapause (Hahn and Denlinger, 2007). The basic fat body cell is the trophocyte, which contains a high density of lipid droplets composed primarily of triglycerides, glycogen, and protein granules (Dean et al., 1985). In order to utilize the stored energy, glycogen is converted into glucose, then released into the hemolymph as trehalose, the primary carbohydrate form in insects, while lipids are mobilized by adipokinetic hormones for use around the body (Mcdougall and Steele, 1988; Thompson, 2003). In preparation

for diapause, feeding activity will increase to build up resources in the fat body (Hahn and Denlinger, 2007). Glycogen and lipid levels act as important resources to the insect as they are necessary to prevent starvation and dehydration, while stored proteins can be converted into usable energy if diapause is prolonged (Karnavar and Nair, 1969; Brent et al., 2013). When terminating diapause, the fat bodies are significantly reduced to support reproduction, meaning that a shift in the concentrations of fats to carbohydrates will take place (Lees, 1955).

Markers indicating diapause have been primarily based on fat body content and the state of the reproductive organs. Hypertrophic fat bodies around reproductive organs and small underdeveloped reproductive organs are characteristic of the insect being in diapause, while lower fat body content and developed reproductive organs indicate that the insect is reproductive (Lees, 1955; Snodgrass, 2003; Villavaso and Snodgrass, 2004). Identification of diapause is currently done through dissection and relies on specimen quality and expertise of the observer. This may cause issues in properly determining whether a *L. lineolaris* specimen is reproductive or in diapause, especially when specimens have desiccated (Lees, 1955; Snodgrass, 2003). *L. lineolaris* have no external phenotypic changes that occur between physiological phases (overwintering vs. reproductive), unlike its close relative, *L. hesperus*, making detection of diapause only based on these internal factors (Capinera, 2001; Brent, 2012).

The objective of this study was to find an assay that allows for the measurement of physiological differences in *L. lineolaris* between their diapausing and reproductive states through lipid and protein quantification. The ultimate goal of this study was to remove potential observer's bias and make the process of determining the physiological phases more accurate and repeatable.

2. Materials and Methods

2.1. Insects

Insects were collected from the Tidewater Agricultural Research and Extension Center in Suffolk VA, USA. Reproductive specimens were caught on daisy fleabane in June of 2022 and 2023, while diapausing specimens were caught off ragweed in November of 2022 and further induced into diapause according to Villavaso and Snodgrass (2004) through subjecting wild caught *L. lineolaris* to diapause inducing photoperiods in incubation chambers. The diapausing adults were fed a combination of ragweeds and green beans during this time. Specimens were euthanized through freezing and kept on ice before being transported to the Molecular Physiology and Toxicology Laboratory in Blacksburg, VA, USA, where they were stored at -80 °C until chemical analysis was performed.

2.2. Reagents

Anthrone (ACS reagent), D-sucrose (Fisher Bioreagent, Pittsburg PA, USA), D-glucose (Fisher Bioreagent, Pittsburg PA, USA), o-phosphoric acid (85%, Supleco, Bellefonte PA, USA), sulfuric acid (95-98%, Spectrum Chemical, New Brunswick NJ, USA), sodium phosphate dibasic heptahydrate, sodium phosphate monobasic monohydrate, Triton™ X-100 (Fisher Bioreagent,

Pittsburg PA, USA), trehalose (>99.5%, Fluka, Morris Plains NJ, USA), vanillin (99%, Alfa Aesar, Ward Hill MA, USA), and vegetable oil were obtained through Fisher Scientific International LLC (Waltham, MA, USA).

2.3. Measurements of total protein, carbohydrates, and lipids.

2.3.1. Tissue Homogenization. Individual diapause or reproductive tarnished plant bugs were weighed and homogenized in 100 µL of ice-cold sodium phosphate (100 mM) buffer (pH 7.8) containing 0.3% Triton X-100. Homogenization was performed in a microcentrifuge tube (1.8 mL) using a Bel-ART® disposable pestle and homogenizer (Fisher, Scientific). Homogenates were centrifuged at 10,000 x *g* for 10 min at 4°C. The supernatant was transferred to a clean tube and used to perform chemical assays.

2.*3.2. Total Protein.* The total protein concentration from individual insect homogenates was assayed using the bicinchoninic acid (BCA) method. A Prometheus BCA Protein Assay Kit (Genesee Scientific, City, State, USA) with a bovine serum albumin (BSA) standard curve was used according to the manufacturer's instructions. Absorbance measurements were made at 562 nm using a SpectraMax M2 multimode microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.3.3. Total Carbohydrate. Total carbohydrate concentration measurements were performed using the Anthrone Reagent (2 g Anthrone per 1 L of sulfuric acid) and a modified procedure from Van Handel and Day (1998). Briefly, an aliquot (25 µL) of the tissue homogenate or carbohydrate standard curve (glucose, sucrose, or trehalose) was added to a 10 x 75 mm borosilicate glass tube (Fisher Scientific), followed by 2 mL of Anthrone Reagent. This mixture was incubated for 15 min at 90 °C then cooled to room temperature. An aliquot (200 µL) was added to a 96-well plate where the absorbance was measured at 625 nm using the SpectraMax M2 multimodal plate reader.

2.3.4. Total Lipids. The total lipid content in insect homogenates was measured using a modified procedure from Van Handel and Day (1998). An aliquot (25 µL) of the homogenate or a known concentration of vegetable oil in chloroform (standard curve) was added to a 10 x 75 mm glass tube containing chloroform (200 µL) and sulfuric acid (200 µL) each. Lipid samples were incubated for 10 min at 90°C then allowed to cool to room temperature, followed by the addition of 2 mL vanillin reagent (600 mg vanillin with 100 mL with 85% phosphoric acid in a 500 mL final volume). Samples were vortexed, and an aliquot (200 µL) was placed in a microtiter plate. The absorbance was measured at 625 nm using the SpectraMax M2 multimodal plate reader.

2.3.5. Data analysis. Data were analyzed using R (Version 4.3.1 R Core Team, 2023) with base R functions. Protein and carbohydrate data were successfully normalized through transformations

using $\log_{10}$ and subjected to a Welsh two sample t-test. Lipid data could not be transformed and remained non-parametric, causing lipid concentrations to be subjected to a Wilcoxon rank-sum test. The lipid and carbohydrate tests were set with an alternative hypothesis of a greater total concentration in the diapausing population. For the protein test, the alternative hypothesis assumed lower protein levels in the diapausing population. Adults subjected to more than one assay type were analyzed across nutrient levels in order to find separation between physiological status.

3. Results

3.1. Protein Analysis

The diapausing population exhibited a lower protein level (174.49 ± 4.57 µg/mg per insect, mean ± SE) compared to the reproductive population (225.53 ± 8.38 µg/mg per insect; **Figure 3.1.**), with the diapausing population demonstrating significantly lower levels ($t = 22$, $df = 32.87$, $p < 0.0001$). Reproductive insects tested displayed a distribution of 146.33 µg/mg of protein per insect ($n = 21$) while diapausing individuals had less variability among the distribution of protein levels (range = 65.70 µg/mg per insect, $n = 14$). Notably, the overall population included four insect specimens overlapping between the two groups.

3.2. Carbohydrate Analysis

The diapausing population exhibited a higher level of carbohydrates (87.69 ± 6.50 µg/mg per insect) compared to the reproductive population (70.44 ± 5.62 µg/mg per insect), with the diapausing population having increased carbohydrate levels (t = 2.317, df = 33.97, p = 0.0133; **Figure 3.2.**). The ranges for the diapausing and reproductive populations were 100.76 µg/mg tissue (n = 14) and 104.95 µg/mg per insect (n = 22) respectively. Across both populations, ten insect specimens overlapped between the two groups.

3.3 Lipid Analysis

The diapausing population exhibited higher lipid levels (1588.40 ± 127.73 µg/mg per insect) compared to the reproductive specimens (754.71 ±38.93 µg/mg per insect), with the diapausing and reproductive populations having significantly different lipid levels (Wilcoxon Rank Sum = 508, n = 48, p < 0.0001; **Figure 3.3.**). The ranges for both populations were at 1930.59 for diapausing specimens (n = 21) and 974.08 for reproductive specimens (n = 28); and six outliers spanning both populations.

3.4 Combining Assay results

All specimens that were subjected to quantification across multiple nutrients were visualized through the pairing of two assay results together to find the distribution between populations across nutrients. The pairing of carbohydrate and lipid quantification assays resulted in two distinct populations with no overlap (**Figure 3.4A.**). Similarly, the combination of lipid and protein

quantification assays exhibited similar results to the combined pairing of carbohydrates and lipids, showing no overlap between specimens and a separation between populations (**Figure 3.4B.**). It is noteworthy that only five reproductive specimens had both carbohydrate and protein levels quantified, indicating the possibility of greater variation within the reproductive population when lipid and protein concentrations are paired. In contrast, once paired, carbohydrate and protein quantification assays exhibited four outliers overlapping between populations with no clear distinction between populations (**Figure 3.4C.**).

4. Discussion

It must be noted that limitations existed with *L. lineolaris* used for this study, as we were unsuccessful in establishing a colony. Therefore, the measurement of nutrients was conducted on two distinct wild populations with reproductive adults collected during the summer on *Erigeron* spp. plants and diapausing adults collected in the winter on *Ambrosia* spp. plants. Differences in nutrient levels could have been influenced by these weed hosts could not be accounted for through inability to standardize diet, as efforts to establish a colony failed.

Within adults tested, reproductive *L. lineolaris* exhibited higher protein levels compared to those that were in diapause. Despite the increase in fat bodies in diapausing individuals, it appears that there was no corresponding increase in protein storage. This may be attributed to the upregulation of hexameric proteins (Burmester 1999), which act as storage for individual amino

acids, as opposed to a general upregulation of proteins during diapause induction. Notably, the overall expenditure and expression of proteins are downregulated during diapause, allowing stored amino acids to be utilized as needed, rather than a substantial increase in stored protein granules (Burmester 1999; Denlinger et al., 2004; Hahn and Denlinger, 2011). The formation of oocytes through the synthesis of complex proteins such as vitellogenin may contribute to differences between diapausing and reproductive females (Engelmann, 1979). However, the influence of sex was not included in this study.

It is commonly observed that diapausing insect species exhibit higher carbohydrate levels than their reproductive counterparts such as in *Ceroplastes japonicus* and *Sarcophaga crassipalpis* (Arrese and Soulages 2010; Michaud and Denlinger 2007; Hahn and Denlinger 2011; Liu et. al, 2017). However, carbohydrates do not consistently serve as the primary energetic nutrient metabolized during diapause and may have an increased roll as a cryoprotectant instead (Zachariassen, 1985). In the case of diapausing *L. lineolaris*, carbohydrate levels were found to be significantly elevated compared to reproductive populations. The separation in distribution of carbohydrates between reproductive and diapausing populations confirm the importance of carbohydrates as an energetic source or cryoprotectant within diapausing *L. lineolaris* (Zachariassen, 1985; Hahn and Denlinger 2011).

Diapausing *L. lineolaris* exhibited higher lipid levels compared to the reproductive population, consistent with similar findings in *Lygus hesperus* as well as other diapausing insect species (Downer and Matthews, 1976; Brent et al., 2013). The significant increase in concentration aligns with the anticipated pattern of elevated lipid levels correlating with hypertrophic fat body

(Lees, 1955; Snodgrass, 2003). The distribution of lipid levels had the greatest variation within the respective populations (diapausing vs. reproductive) and among the three nutrients tested in this study. However, even with the high variation of lipid levels, concentrations in diapausing and reproductive populations had divergent means and did not overlap in SEM, indicating an importance of the upregulation of lipids within diapausing individuals. In the diapausing population, fluctuations in lipid concentration may serve as an indicator of the health, host quality, and survivability of overwintering individuals. *L. lineolaris* may have not been able to store lipids as effectively if pre-diapause hosts were poor. While in diapause, when nutrients are not available, *L. lineolaris* may have to rely on lipids as energetic resources which can deplete stores and cause a decrease in usable energy stores and ability to tolerate the cold (Hahn and Denlinger 2011).

When pairing assay results, a reduction in variation between physiological status (diapausing vs. reproductive) was observed, however, only individuals which were run across assays were included in these results. Assay pairings including lipid concentrations best separated physiological status in adults that were analyzed across multiple assays. The pairing of carbohydrates and protein assays was not successful at separating *L. lineolaris* by status despite correlations seen in Deans et. al. 2019, between carbohydrate and protein levels in reproductive *Lygus* diet selection. Click or tap here to enter text.This suggests that utilizing carbohydrate and protein quantification assays in combination may not yield better results than employing lipid or protein quantification alone. The combination of these assays may offer an alternative method to determining physiological status in *L. lineolaris*. Furthermore, if specimens were combined and used in a collective homogenate for the use in the combined lipid and protein or carbohydrate and

lipid quantification assays, it may be possible to determine the ratio of diapausing individuals in a population, through the establishment of a known diapause ratio standard curve. This could offer both the increase and accuracy of diapause determination while conserving time compare to dissecting or performing assays individually. This could potentially allow studies on the diapause status of *L. lineolaris* to be expanded to consider greater population sizes or increased subpopulations included.

In conclusion, this study revealed difference on nutrient concentrations between diapausing and reproductive populations of *Lygus lineolaris*. Specifically, diapausing individuals exhibited higher overall carbohydrate and lipid levels, but lower overall protein levels compared to their reproductive counterparts. It is suggested that lipid and protein quantification assays may effectively serve to infer the reproductive status in *L. lineolaris*. Furthermore, the combined use of carbohydrate and lipid or lipid and protein quantification assays hold even greater potential, by offering an alternative method for status determination for *L. lineolaris* individuals and whole populations. These nutrient assays could be extended to assess reproductive status in other insect species, showcasing its broader applicability.

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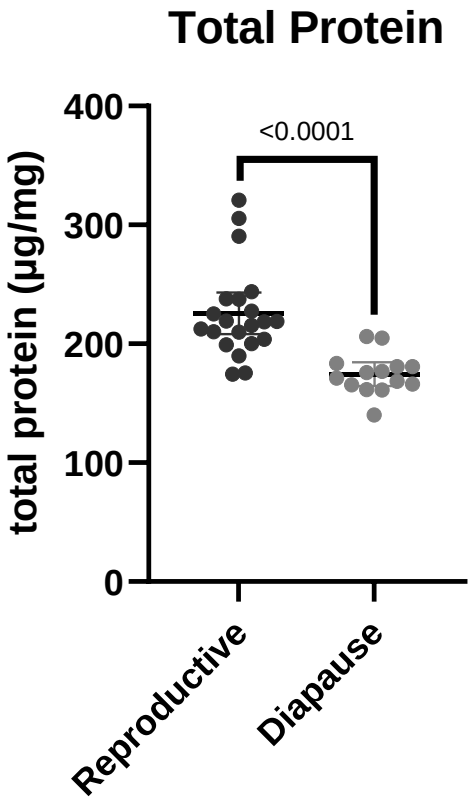

Figure 3.1. Comparison of proteins between reproductive and diapausing *Lygus lineolaris* specimens collected from fleabane and ragweed during the summers and winters of 2022- 2023 in Suffolk VA. Protein was quantified and recorded as µg/mg per insect ± SEM. Horizontal bar among data points denotes the mean concentration.

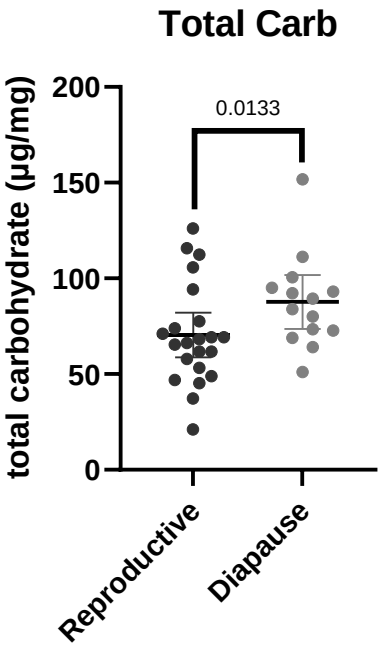

Figure 3.2. Comparison of carbohydrates between reproductive and diapausing *Lygus lineolaris* specimens collected from fleabane and ragweed during the summers and winters of 2022- 2023 in Suffolk VA. . Carbohydrates were quantified and recorded at µg/mg per insect ± SEM.

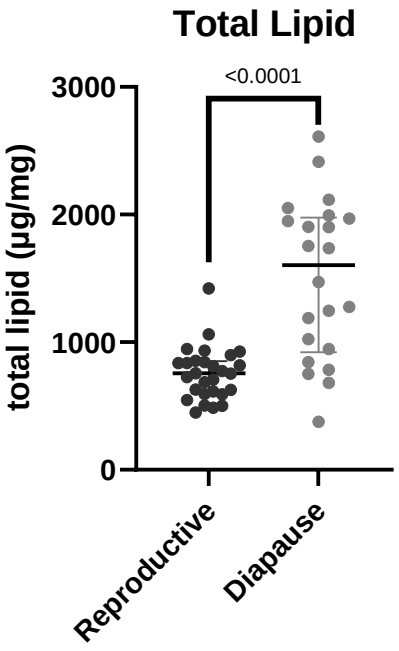

Figure 3.3. Comparison of lipids between reproductive and diapausing *Lygus lineolaris* specimens collected from fleabane and ragweed during the summers and winters of 2022- 2023 in Suffolk VA. Lipids were quantified and recorded as µg/mg per insect ± SEM.

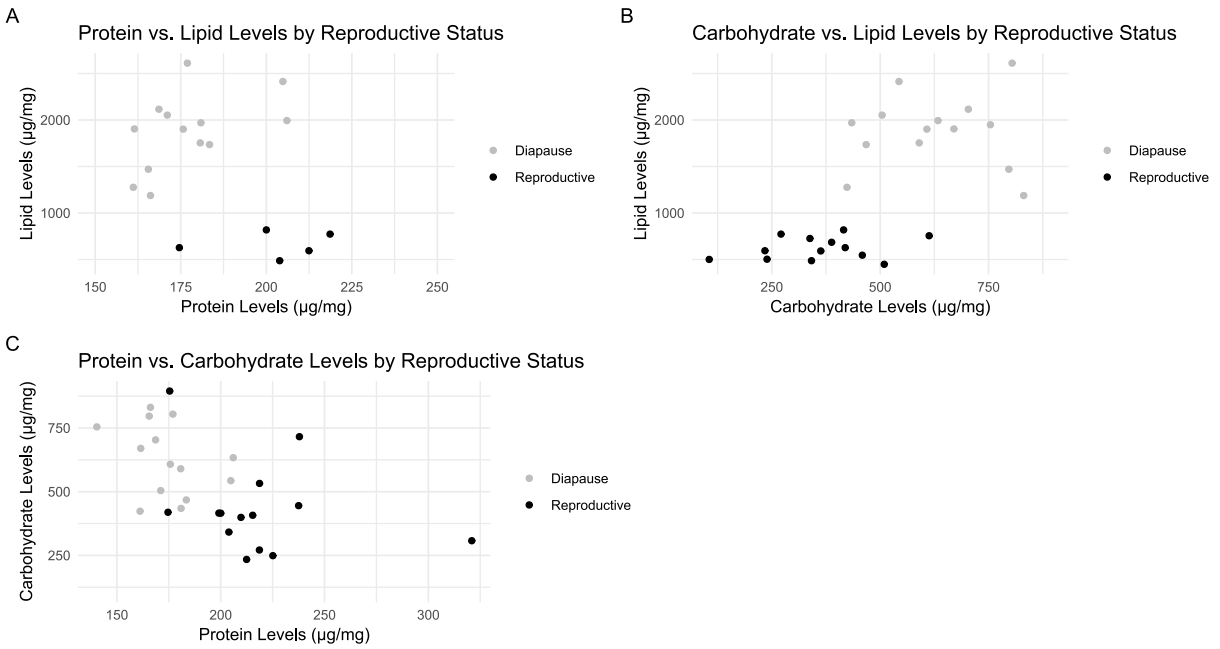

Figure 3.4. Cross analysis of diapausing and reproductive *L. lineolaris* individuals subjected to **A)** carbohydrate and lipid quantification assays, **B)** protein and lipid quantification assays, and **C)** protein and carbohydrate quantification assays. Units are presented as µg/mg per insect.

Chapter 4

Flight potential of *Lygus lineolaris* (Hemiptera: Miridae) influenced by weeds in mid-Atlantic cotton fields

Abstract. The tarnished plant bug, *Lygus lineolaris* (Palisot de Beauvois), is a key cotton pest of mid-Atlantic cotton. In the spring, *L. lineolaris* disperses on a variety of weedy hosts, before transitioning to cotton in the mid-summer following weed senescence. The flight potential for *L. lineolaris* could influence how individuals disperse into cotton fields. This study determined the impacts of weed hosts on the ability of *L. lineolaris* to flight under controlled conditions. Adult populations were collected from daisy fleabane, cutleaf evening primrose, and curly dock weeds, and analyzed to find differences in flight capacity through the use of a flight mill, body weight, caged behavioral assays, and nutrient (carbohydrate, lipid and protein) quantification assays. Flight capacity was not found to differ between populations tested while mortality on the flight mill was highest in the starved control population. The starved control and daisy fleabane populations had the lowest body weight and lipid concentrations. Carbohydrates were statistically uniform across populations of *L. lineolaris*, while protein concentrations adults from the starved control had higher protein levels than the populations coming from plant hosts. Behavioral analyses exhibited higher rates of activity in cages with *L. lineolaris* coming from plant hosts compared to the starved control. This study suggests that host, nutrient, and weight differences are unlikely to impact flight potential of *L. lineolaris*, while inferring that the loss of a host may lead to an increase of a dispersing into crops such as cotton.

Key Words: flight mill, tarnished plant bug, weed host, nutrition, diel activity

Introduction

The tarnished plant bug, *Lygus lineolaris* Palisot de Beauvois (Hemiptera: Miridae), is an abundant polyphagous pest in the Mid-Atlantic United States, known to feed on a diverse range of weedy and cultivated hosts. With a documented feeding history on over 300 plant species across North America, it stands as a significant economic threat to a variety of crops, including cotton (*Gossypium hirsutum* L., Malvaceae) (Ridgway and Gyrisco, 1960; Cleveland, 1982; Layton, 2000). During winter, *L. lineolaris* enters a state of diapause on plant debris or dormant hosts, resuming activity on weedy hosts from early spring through mid-summer (Cleveland, 1982; Snodgrass, 2003). Common weed hosts during the spring and early summer include daisy fleabane (*Erigeron annuus*), cutleaf evening primrose (*Oenothera laciniata*), and curly dock (*Remux crispus*), among others (Snodgrass et al., 1984; Young, 1986; Esquivel and Mowery 2007). When these weedy hosts senesce, *L. lineolaris* disperses to cotton fields, where its feeding on meristematic tissue often results in substantial plant injury and economic losses (Snodgrass et al., 1984; Fleischer et al., 1988; Layton, 2000; Outward et al., 2008).

Infestation patterns and the distribution of *L. lineolaris* within cotton fields remain poorly understood. Although population dynamics have been correlated with temperature, degree days, and proximity to other host crops such as corn, the predictive power of these factors appears limited (Kumar and Musser, 2009; D'Ambrosio et al., 2019; Dorman et al., 2020). Nearby weed

hosts also play a direct role in influencing such population dynamics, with fields surrounded by weeds more prone to heavier adult infestations compared to those without immediate weed hosts nearby (Fleischer et al., 1988; D'Ambrosio et al., 2019). However, *L. lineolaris* dispersal into cotton occurs even from distant weed hosts, likely due to the ability of this insect to transition from recently senesced or destroyed weeds to other host patches (Snodgrass et al., 2006; Outward et al., 2008). This dispersal behavior suggests the ease with which *L. lineolaris* disperse and moves between host patches, limiting the efficacy of general cultural control efforts surrounding cotton fields (Cleveland, 1982; Fleischer et al., 1988; Snodgrass et al., 2003, Abel et al., 2007; Outward et al., 2008).

The dispersal and movement of *L. lineolaris* between hosts is dependent on the insect's ability to fly. Insect flight, especially during dispersal or migration events, demands the recruitment of various energetic substrates by flight muscles. Carbohydrates such as trehalose, glucose, and glycogen serve as primary energy sources in short flights and are the first energetic sources used during starvation (Beenakker et al., 1984; Zhang et al., 2019). During periods of extended flight and starvation, adipokinetic hormone gets released to mobilize lipids from the fat bodies increasing available energy for sustained flight activities (Staubli et al., 2002; Gergs and Jager, 2013). In many insect species, lipids act as the primary energy source for both long-distance flight and flight during periods of starvation (Beenakker et al., 1984; Chapman, 1988; Ryan and van der Horst, 2000). Proline, oxidized in the citric acid cycle, contributes to the energy used for takeoff and is a primary energy source for certain adults (Beenakker et al.,

1984). Proline can be broken down from stored proteins during starvation, providing usable energy to the insect (Auerswald and Gäde, 2000).

Flight mill studies offer insights into insect flight capacity under controlled conditions in a laboratory environment. These studies typically assess the impact of body weight, sex, age, reproductive status, and environmental conditions on laboratory-bred or wild populations of a selected species (Naranjo, 2019; Babu et al., 2020). Previous studies on *L. lineolaris* and similar species have established a baseline for understanding the flight potential of these individuals (Stewart and Gaylor, 1994; Blackmer et al., 2004). Stewart and Gaylor (1994) discovered that early reproductive male and female *L. lineolaris* exhibit the greatest ability to colonize new food sources, such as mid-summer cotton, due to their inclination toward long flights. Environmental stimuli, including time of day, photoperiod, and light conditions have also been shown to influence *L. lineolaris* flight activity and orientation (Blackmer et al., 2004). Flight potential studies on other hemipterans, such as *Halyomorpha halys* Stal (brown marmorated stink bug, Hemiptera: Pentatomidae) gave a foundation on the influence of environmental factors, diapause status and collection date (Lee & Leskey, 2015; Wiman et al., 2015). While baseline studies exist for *L. lineolaris* flight, additional knowledge is required to comprehend the ability of this insect to disperse between weed and cultivated hosts in mid-summer. This study aimed to investigate the flight capacity of *L. lineolaris* originated from certain weed hosts. The flight capacity will be documented by running a flight analysis using a flight mill, nutrient quantification, and behavior assay, to identify the role for these selected weed hosts in relationship to *L. lineolaris* dispersal capabilities.

Materials and Methods

Flight analysis using flight mills

In 2022, wild adult *L. lineolaris* were captured from weed hosts on July 20 through 23 at the Tidewater Agricultural Research and Extension Center (Tidewater AREC) in Suffolk, VA, with a total of 96 adults flown. In 2023, collection sites were the Tidewater AREC and two farms in Wakefield, VA. Collections of these adults alternated weekly between locations from June 6 to July 13, prompted by weed host senescence, with a total of 759 adults flown. Targeted weeds to be used as treatments included 1) daisy fleabane, 2) cutleaf evening primrose, and 3) curly dock, identified by Snodgrass (1984) as optimal *L. lineolaris* weed hosts from late spring to early summer. The control was taken from daisy fleabane, which is considered one of the most preferred summer weed hosts (Cleveland, 1982; Snodgrass et al., 1984). Control adults were then starved with only tap water for 24-h before undergoing preparation for the flight, replicating host destruction and plant senesce. The period of 24-h was selected as adults could be easily caught alongside the populations subjected to the flight mill the day previous. Adults were collected using sweep nets and aspirators, placed into 30.5 x 30.5 x 30.5 cm (12 x 12 x 12 inch) mesh cages, and transported to the laboratory. A total of 224 adults were collected and flown for the control, 219 for daisy fleabane, 224 for evening primrose, and 187 for curly dock. Replicate was defined as week sampled.

The flight mill system used for this project consisted of 32 pivot arms with a 10-cm radius, adhering to the assembly procedure detailed by Attisano et al. (2015). To reduce weight, modifications were made to the arm structure using 36-gauge metal wire, and the side of the arm attaching to the adult was made detachable for tethering (**Figure 4.1.**). This study also followed the protocol outlined by Attisano et al. (2015) for operating the flight mill, and for employing an electricity data logger combined with WINDAQ software (version 1.32.0, DATAQ Instruments Inc., Akron, OH). Data standardization and analysis of results from the WINDAQ files were carried out using Python scripts (Python Co., Wilmington, DE). Flight sessions in 2022 had a duration of 8 h, but were extended to 22 h in 2023 to maximize flight session, while still allow for sessions be run on back to back days. The room housing the flight mill had a temperature of 22°C ± 2°C, with redirected airflow from the air conditioning unit and physical barriers to block natural light. Illumination was provided by two 40-watt equivalent LED cool white tube lights positioned directly above the flight mill. Bulbs were chosen to prevent effects via the flicker flight frequency by flickering above insect light sensitivity through best mimicking natural light (Shields 1989). *Lygus lineolaris* subjected to an 8-hour flight mill session experienced a constant photoperiod, while those undergoing a 22-h session had a photoperiod of 12:10 (L:D).

To prepare individuals for tethering to the flight mill arms, adults were randomly selected from the collected populations and checked for any damage incurred during the collection process. Individual adults were weighed, recording to the nearest 0.0001 g. Adults were briefly anesthetized at 4°C for a maximum of 10 minutes prior to tethering. Adults were tethered directly to the end of the detachable side of the arm using Stick-um Candle Adhesive (Fox Run,

Warminster PA, USA). Flight mill sessions commenced between 12:00 and 14:00 on the day of collection or, in the case of the control group, the day after. Tethered adults remained undisturbed throughout the session. Following the session, adults were assessed for vitality by their ability to walk when prompted, and living *L. lineolaris* were weighed. Dead adults could not be accurately weighed due to desiccation. All adults were sexed by checking for the presence of an ovipositor upon removal from the flight mill.

Nutrient Quantification

Lygus lineolaris adults were collected directly from daisy fleabane, cutleaf evening primrose, and curly dock, and were euthanized immediately, with the control being caught off of daisy fleabane and starved for 24-h in the presence of tap water before being euthanized. Adults were collected during the week of 20 July 2022 in Suffolk, VA and throughout June 2023 in Suffolk and Wakefield, VA, with site and site/year used as the replicate. Individuals were euthanized by being placed in a -20°C freezer for at least ten minutes.

Lygus lineolaris adults were homogenized in a microfuge tube (1.8 mL) with 100 µL of ice-cold sodium phosphate buffer (100 mM, pH 7.8) containing 0.3% Triton^{TM} X-100 (Fisher Bioreagent, Pittsburg PA, USA). Homogenization was carried out using a Bel-Art® disposable pestle and homogenizer (Fisher Scientific, Waltman MA, USA). The homogenates underwent centrifugation at 10,000 RPM x *g* for 10 minutes at 4°C. The resulting supernatant was transferred to a clean microfuge tube for nutrient quantification in all three biochemical assays.

Total carbohydrate concentration was quantified using an Anthrone Reagent composed of 2 g Anthrone (ACS reagent) to 1 L sulfuric acid (95-98% sulfur, Spectrum Chemical, New Brunswick NJ, USA), and following a modified procedure from Van Handel and Day (1998). An aliquot of 25 µL from each tissue homogenate was mixed with 2 mL of the Anthrone Reagent. After incubation for 15 min at 90°C and cooling to room temperature, 200 µL of each mixture was transferred to a 96-well plate, where an absorbance was measured at 625 nm using a SpectraMax M2 multimode microplate reader (Manufacturer information). A total of 129 *L. lineolaris* adults were used across two years and four treatments (three populations collected from weedy hosts and a control). Of those *L. lineolaris* tested, 37 adults were from the control, 37 from daisy fleabane, 35 from cutleaf evening primrose, and 20 from curly dock weeds.

Total lipid concentration was measured through a modified procedure from Van Handel and Day (1988). An aliquot of 25 µL from each tissue homogenate was mixed with 200 µL of chloroform and 200 µL of sulfuric acid in a 10 x 75 mm borosilate glass tube (Fisher Scientific, Waltman MA, USA). This process was repeated with a vegetable oil (Fisher Scientific, Waltman MA, USA) standard curve. Samples were incubated for 10 min at 90°C and cooled to room temperature, followed by the addition of 2 mL of a vanillin reagent (600 mg vanillin and 100 mL 85% phosphoric acid in a 500 mL final volume). After vortexing, 200 µL of each mixture was transferred to a 96-well plate, and absorbance was measured at 625 nm using a SpectraMax M2 multimodal plate reader. A total of 131 adults were assayed across two years and four treatments. Of those adults tested, 37 were from the control, 37 from daisy fleabane, 37 from cutleaf evening primrose, and 20 from curly dock weeds.

Total protein concentration in individual adults was quantified using the bicinchoninic acid (BCA) method with a Prometheus BCA Protein Assay Kit (Genesee Scientific, El Cajon, USA), and then compared against a bovine serum albumin (BSA) standard curve made by following the manufacturers procedures. Absorbance measurements were taken at 562 nm using a SpectraMax M2 multimode microplate reader (Molecular Devices, Sunnyvale, CA, USA). A total of 88 *L. lineolaris* adults were assayed across two years and four treatments, with 22 adults assayed for each treatment.

Behavior Assay

Cotton plants were sourced from an untreated field planted with DeltaPine 1646 B2XF with Acceleron Basic (ADNF) seed treatment (Bayer Crop Science, St. Louis MO, USA). Squaring cotton plants (at the beginning of blooming), obtained during the 9-12 node stage, were collected from a field located at the Tidewater AREC on 12 July, 18 July, and 28 July 2023. These plants were subsequently transplanted into 23 cm (9 inch) plastic pots, each covered with a 30.5 x 30.5 x 30.5 cm mesh cage. In the greenhouse, four cages with one cotton plant per cage, were established for each of the treatments consisting of 1) a control, 2) daisy fleabane, and 3) cutleaf evening primrose.

Lygus lineolaris adults were collected from daisy fleabane and cutleaf evening primrose on the same morning as cotton plants. Control adults were collected the morning before from daisy fleabane and fed only tap water. Each cage received a total of 15 *L. lineolaris* adults, introduced at either 10:00 or 12:00 on the starting day of each of the four replicates consisting of separate cages with only three replicates included in the final assay. Observations commenced at

the 2-hour mark after the release of selected adults, with subsequent observations every 2 hours, spanning a total of 24 hours. At each observation time, behavior was documented during two 1-minute increments, as number of specimens performing a specific behavior. Recorded behaviors included the number of adults flying, and walking, as well as number of adults on cotton plants.

Data Analysis

Data was analyzed using R (Version 4.3.1 R Core Team, 2023), and executing the 'lme4' (Bates et. al., 2015), 'car' (Fox et. al., 2019), 'emmeans' (Lenth, 2018) and 'multicomp' (Hothorm et. al. 2023) packages. Recorded flight data of flight time (s), flight distance (m), starting weight (g), and ending weight (g) were normalized using a log-transformation ($\log_{10} + 0.1$), while percent weight change was normalized using a square root transformation. All models were subjected to a linear mixed model. Average flight speed (m/s), number of flight events, flight mill survival (all), and flight mill survival (only fliers) were fitted to generalized linear mixed models (GLMM) with a logarithmic, Poisson, and binomial distributions respectively. The fixed effects were weed host, sex, and the interaction weed host and sex, with location nested with sampling week was included as the random effect in all models. Nutrition quantification was subjected to a logarithmic distributed GLMM with weed host, sex and their interaction as the fixed effects, and location nested with replicate of site/year as the random effect. Behavior assay data was subjected to a Poisson distributed GLMM, with weed host and observation time as the fixed effects, and cage and assay date as random effect. All linear models were calculated using the 'lme4' package. Summary statistics were calculated using the 'car' package. Mean separations were subjected to a Tukey's post-hoc test with an $\alpha = 0.05$ using the 'emmeans'

package. Compact letter assigned to all post-hoc mean separations and confidence intervals (CI) were displayed executing the 'multicomp' package.

Results

Flight analysis using flight mills

Total flight time (s) of *L. lineolaris* adults was not influenced by weed hosts (df = 3, 72.28, F = 0.31, P = 0.8176) across sampling weeks. The impact of sex on flight time was not significant when comparing the model results (df = 1, 73.81, F = 4.22, P = 0.0436), to the Tukey's post hoc (95% CI: -0.53 to 0.00; t = -1.97, p = 0.0525), as no pairwise comparisons were significant. The interaction between weed host and sex was not significant (df = 1, 72.13, F = 0.66, P = 0.5780). Similarly, flight distance (m) of *L. lineolaris* adults was not influenced by weed hosts (df = 3, 72.85; F = 0.31, P = 0.8149), while also not having any difference due to sex (df = 1, 74.06; F = 3.61, P = 0.0614) or the interaction between weed host and sex (df = 3, 72.52; F = 0.80, P = 0.5004). Average speed (m/s) was similar across populations exhibiting no change due to weed host (χ^2 = 3.51, df = 3, P = 0.3189), sex (χ^2 = 0.83, df = 1, P = 0.3629), or the interaction between the two terms (χ^2 = 4.87, df = 3, P = 0.1812). Despite the lack of association between these flight parameters and weed hosts, flight events for *L. lineolaris* adults during the flight sessions were affected by interaction between weed host and sex (χ^2 = 11.44, df = 3, P = 0.0096; **Figure 4.2.**), with the highest number of events occurring in males and females from control populations, and the lowest in adults from curly dock and males from cutleaf evening primrose.

Starting weight (g) of *L. lineolaris* adults was influenced by weed hosts (χ^2 = 15.85, df = 3, P = 0.0012; **Figure 4.3.**), with the highest weights documented from adults collected from cutleaf evening primrose, and higher weights from curly dock when compared to the control. Sex (χ^2 = 0.82, df = 1, P = 0.3639) and the interaction between weed host and sex (χ^2 = 0.75, df = 3, P = 0.8603) did not have an impact on starting weight. Ending weight of *L. lineolaris* adults was not linked to weed hosts (χ^2 = 7.72, df = 3, P = 0.0521**)**, or linked to sex (χ^2 = 0.05, df = 1, P = 0.8250). Interactions between weed host and sex was not significant (χ^2 = 5.31, df = 3, P = 0.1626**)**. Percent change in weight of *L. lineolaris* subjected to the flight mill was not impacted by weed host (χ^2 = 0.95, df = 3, P = 0.8135**)**, sex (χ^2 = 0.58, df = 1, P = 0.4465), or the interaction between weed host and sex (χ^2 = 4.00, df = 3, P = 0.2615).

Overall survival on the flight mill was impacted by weed hosts (χ^2 = 12.90, df = 3, P = 0.0049; **Figure 4.4.**), with adults coming from the control having lower survival than the treatments. In addition, sex influenced flight mill survival (χ^2 = 5.16, df = 1, P = 0.0231), with males having a survival rate of 36 ± 9%, and female at a rate of 41 ± 7% (95%-CI: 1.40 to 3.15; z = 3.58, P = 0.0003). The interaction between weed host and sex did not impact survival (χ^2 = 3.44, df = 3, P = 0.3280). The survival of adults that flew on the flight mill (flyers only) was not impacted by weed hosts (χ^2 = 4.79, df = 3, P = 0.1876), or sex (χ^2 = 0.98, df = 1, P = 0.3220). The interaction between weed host and sex was not significant (χ^2 = 0.79, df = 3, P = 0.8516).

Nutrient Quantification

Concentrations of carbohydrates were not influenced by weed hosts on *L. lineolaris* adults (χ^2 = 6.90, df = 3, P = 0.0752), or by sex (χ^2 = 0.55, df = 1, P = 0.4602). The interaction

between weed host and sex did not influence carbohydrate levels (χ^2 = 0.61, df = 3, P = 0.8948). Lipid levels for *L. Lineolaris* adults were impacted by weed hosts (χ^2 = 22.58 df = 3, P < 0.0001; **Figure 4.5A.**), but not sex (χ^2 = 2.70, df = 1, P = 0.1005). The interaction between weed and sex did not impact lipid levels (χ^2 = 0.62, df = 3, P = 0.8918). The control adults had the lowest lipid level, followed by daisy fleabane, curly dock, and cutleaf evening primrose. The protein concentration from the adults was influenced by weed host (χ^2 = 8.23, df = 3, P = 0.0415; **Figure 4.5B.**), where control adults had the highest protein concentration, compared to the populations coming off of daisy fleabane, cutleaf evening primrose, and curly dock. Protein levels in *L. lineolaris* adults was significant by sex in the model (χ^2 = 4.16, df = 1, P = 0.0414), while the results of the Tukey's post-hoc analysis (95% CI: 0.94 to 1.04; z = -0.39, p = 0.6981) lacked significant pairwise comparisons. Interaction between weed host and sex was not significant (χ^2 = 7.10, df = 3, P = 0.0688).

Behavior Analysis

Walking behavior was directly influenced by weed host (χ^2 = 10.67, df = 2, P = 0.0048; **Figure 4.6A.**). Adults from cutleaf evening primrose and daisy fleabane had rates of 2.92 and 2.79 adults walking per observation respectively, which were higher than the 2.03 rate of adults walking per observation of the control. The ratio of adults walking on plants significantly change over the observation time periods (χ^2 = 30.21, df = 11, P = 0.0015; **Figure 4.7.**). Overall walking activity increased from the first observation period at 14:00 and peaked at 20:00, while dropping off from 04:00 until the end of the observation periods at 12:00. The interaction between weed host and observation time was not significant (χ^2 = 27.18, df = 22, P = 0.2042). Similarly, flying

behavior of adults was impacted by weed host (χ^2 = 17.17, df = 2, P = 0.0002; **Figure 4.6B.**). Trends of flight between treatments followed activity levels seen in walking behavior, with flight rates of 1.04 adults flying per observation in cutleaf evening primrose and 0.94 adults flying per observation in daisy fleabane compared to the rate of 0.36 adults flying per observation on the control. The ratio of adults flying was not influenced by observation time (χ^2 = 50.41, df = 11, P < 0.0001; **Figure 4.7.**). Flight behavior was highest at the 14:00 and 16:00 observation periods, while slowly decreasing around 06:00, having a slight increase in flight activity in the following observation periods. The relationship between weed host and observation time was not significant (χ^2 = 27.38, df = 22, P = 0.1970). *Lygus lineolaris* that were on the cotton plant sedentary (not walking) within the cage were not impacted by weed host (χ^2 = 4.94, df = 2, P = 0.0844), or the time of observation (χ^2 = 19.09, df = 11, P = 0.0595). Interactions between weed host and time of observation were not significant (χ^2 = 23.13, df = 22, P = 0.3943).

Discussion

The results of this study provided insight into how weed hosts may influence the flight potential of *L. lineolaris* adults to disperse to cultivated cotton. Adult *L. lineolaris* begin the summers on weed hosts before transitioning to cotton plants in mid-late July, leading to their status as a major economic pest of crops in the mid-Atlantic (Cleveland, 1982; Fleischer et al., 1988; Williams, 2017). Weed hosts are commonly found in field edges and road ditches, but even when large scale weed control measures are taken, cotton fields can still experience heavy infestations of *L. lineolaris* in the mid-late summer (Snodgrass et. al., 2003; Snodgrass et al., 2006). To better

understand this insect's ability to disperse throughout the landscape, variables that impacted flight capacity in *L. lineolaris* were studied in populations from common weed hosts. In flight mill assays, males that had been withheld from food before flying were more likely to attempt sustained flights compared to populations coming from weeds. These starved adults were also more likely to have a lower starting body weight compared to weed populations. Laboratory studies focused on measuring nutrient quantification found differences in lipids, and proteins between starved and populations coming from non-crop plant hosts. Analysis of behavior displayed differences between *L. lineolaris* populations taken directly from weed hosts and starved adults, with higher walking and flying activity in adults from weed hosts.

In flight mill assays, higher flight potential was expected to be documented from populations coming from the most preferred host plants. During this experiment, 565 adult *L. lineolaris* were flown, with only 18.1% of those having usable flights. Flights were deemed unusable if recorded flight parameters indicated that flight mill arm got stuck in the sensor, or an insect fell off. The high proportion of non-flyer adults from this study also corresponded with previous research performed on *L. lineolaris* by Stewart and Gaylor (1994), and Blackmer et al. (2004). Differences in flight time, distance, and speed between the control and weed populations were not enough to detect significance. Mortality on the flight mill may have impacted flight results, as the control population experienced 77.3% mortality, significantly higher than the mortality seen from the daisy fleabane, cutleaf evening primrose, and curly dock at 56.8%, 59.8 and 49%, respectively. While there was no difference in the mortality of fliers, overall mortality of the control may still be an indicator of potential methodological issues on the flight mill. The

high number of flight events of males from the control could potentially be due to a heightened flying behavior, which is sometimes observed in insects when a food source becomes poor or has been removed, similar to field settings with adult *L.* lineolaris infesting cotton after weed senesce (Fleischer et al., 1988; Scharf 2016). Female *L. lineolaris* have been found to more regularly colonize new plant hosts (Stewart and Gaylor, 1994), and this may have been one of the driving factors where females were overall less effected by host compared to males.

There were differences in body weight of flying *L. lineolaris* between populations collected from experimental treatments. Populations from cutleaf evening primrose and curly dock had higher pre-flight weights, compared to the control, while adults collected from daisy fleabane did not vary in pre-flight weight from the other populations. Increased pre-flight body weight has been found to increase flight capacity in some insect species (Evenden et al., 2014; Makumbe et al., 2020), but effects of pre-flight weight were not confirmed in this study. Post-flight weight of surviving adults was not different between populations, as weights became more uniform by the end of the flight mill sessions. Sex was not correlated to pre-flight weight, but differences between sexes appeared post-flight. Females are expected to be larger due to gender dimorphism commonly seen in adult insects (Forrest, 1987; Colwell, 2000; King et al., 2018), and the stress of flight could have made these differences more apparent. The resulting weight loss during flight was uniform across populations and also bringing the post-flight weight back to the overall mean. While weight loss was constant, there appears to be a general floor which *L. lineolaris* fliers reached during the flight mill session.

Nutrition levels at the preflight status from populations coming directly off weeds were believed to potentially impact flight ability of *L. lineolaris* adults. Carbohydrate levels were uniform across populations, as these concentrations were sustained well enough in the starved control to prevent. Carbohydrates are likely to have impacted short distance flight and possible had an effect on long distance flight, as there is the possibility carbohydrates are the primary energetic source in both cases (Auerswald and Gäde, 2000). Lipid levels were elevated in populations from cutleaf evening primrose and curly dock, compared to the control where lipid levels were depleted during the 24-h withheld from a food source. Therefore, populations coming directly from cutleaf evening primrose and curly dock may have higher energetic resources in the form of lipids to put towards long distance flight (Beenakker et al., 1984; Ryan and van der Horst, 2000), while adults in the control population most likely had to metabolized lipid stores to maintain carbohydrate concentrations within the hemolymph (Arrese and Soulages, 2010). Protein appeared to increase in the starved control adults, and were significantly higher than individuals coming from weed hosts. The increase in protein concentration in starved *L. lineolaris* may be due to stressful conditions such as dehydration and increased lipid metabolism, causing the upregulation in transcription of protective proteins (Marron et al., 2003; Tamang et al., 2022), and not related to any flight event.

Another goal of this study was to pair the flight potential of *L. lineolaris* adults collected from weed hosts to the observed behavior when put in contact with a squaring cotton plant. Overall activity trends were consistent with flight activity of *L. lineolaris* seen in the study conducted by Blackmer et al. (2015), which measured flight mill activity over time without

considering host. Activity levels were higher during the first day compared to the second, as adults settled and most likely began feeding as time continued This may also be similar to the response that *L. lineolaris* have following weed senesce or host destruction, as they adjust to the lack of weed hosts to disperse to cotton (Snodgrass et. al., 1984; Fleischer and Gaylor, 1988). Lowering of overall activity was not likely due to starvation alone as flight mill sessions indicated increased flight events in control populations compared to some populations taken off of weeds. This reduced activity was potentially due to the presence of a food source and harborage side, which reduced the stimulation to move leading to a prioritization in feeding and energy conservation. *L. lineolaris* coming directly from weed hosts may have not shown such behavior due to individuals being able to rely on their energetic reserves. Dispersal mechanisms into cotton are often attributed to weed senesce (Fleischer et al., 1988; Outward et al., 2008), and the behavior seen appears to support the existence of this mechanism. Additionally, dispersing to cotton may not be the first choice of *L. lineolaris* coming from weed hosts, and they may continue to seek out, if other weed hosts are still available in the landscape (Cleveland, 1982; Snodgrass et al., 1984; Fleischer et al., 1988; Esquivel and Mowery 2007).

In conclusion, findings from this study indicated that *L. lineolaris* was able to maintain consistent carbohydrate and protein levels from key early summer weed hosts, while lipid concentrations and body weight were more prone to fluctuate. When *L. lineolaris* were subjected to 24-h starvation, protein levels were elevated, presumably as a protective response to adverse conditions. Other resources measured as lipid concentrations and bodyweight declined during brief periods of starvation. As these energetic resources diminish, there could be an increase in

the flight responses in *L. lineolaris*, directly associated to the dispersion from weeds to cotton at weed senesce. Flight events on a flight mill resulted in elevated flight events in starved individuals. When the same populations were introduced to a cotton plant, flight and activity responses of the starved adults decreased, compared to the activity observed form populations originating from weed hosts. This suggests *L. lineolaris* may disperse to a non-preferred host such as cotton, rather than search out more favorable hosts. In summary, the flight response of *L. lineolaris* appears directly influenced by the results of mid-summer weed senescence, while measurements of total flight potential such as flight distance, time and speed do not appear to be influenced by weed host.

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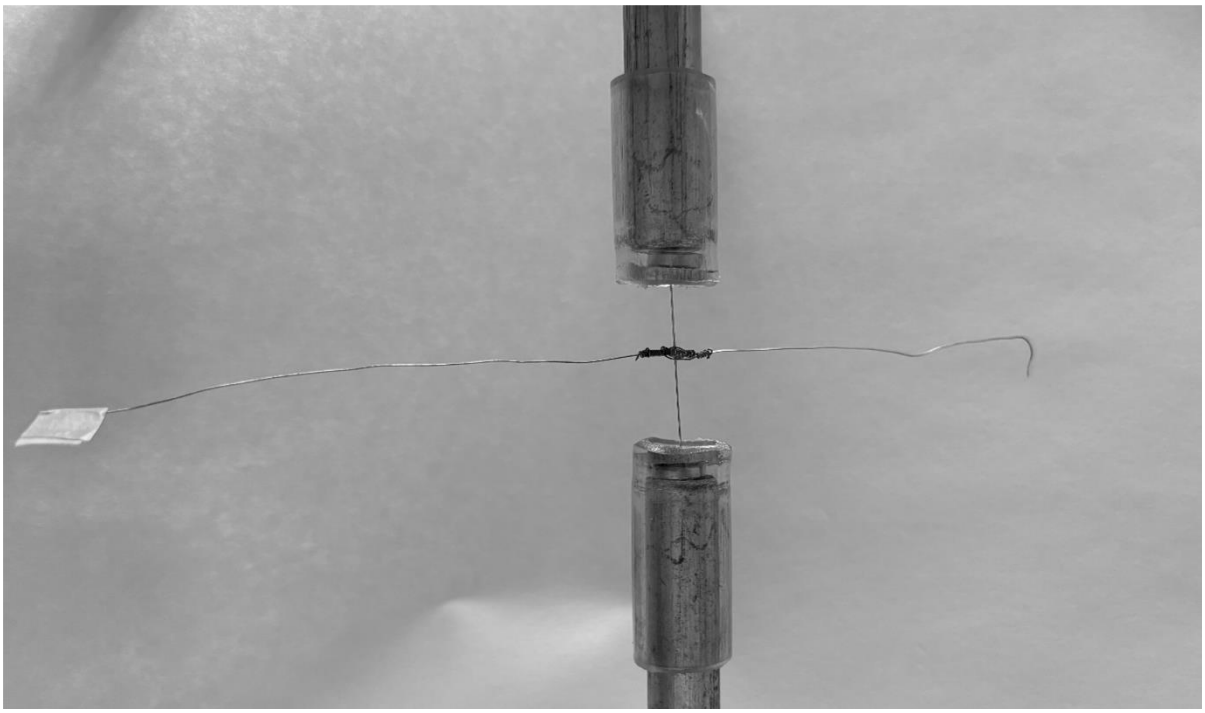

Figure 4.1. Flight mill rotating arm was suspended between two magnets with the center pin formed out of a size 2 stainless steel insect pin. Horizontally a tightly wound 36-gauge wire was stuck through the pin and attached with crazy glue. Each arm was a separate, removable piece made from 36-gauge wire.

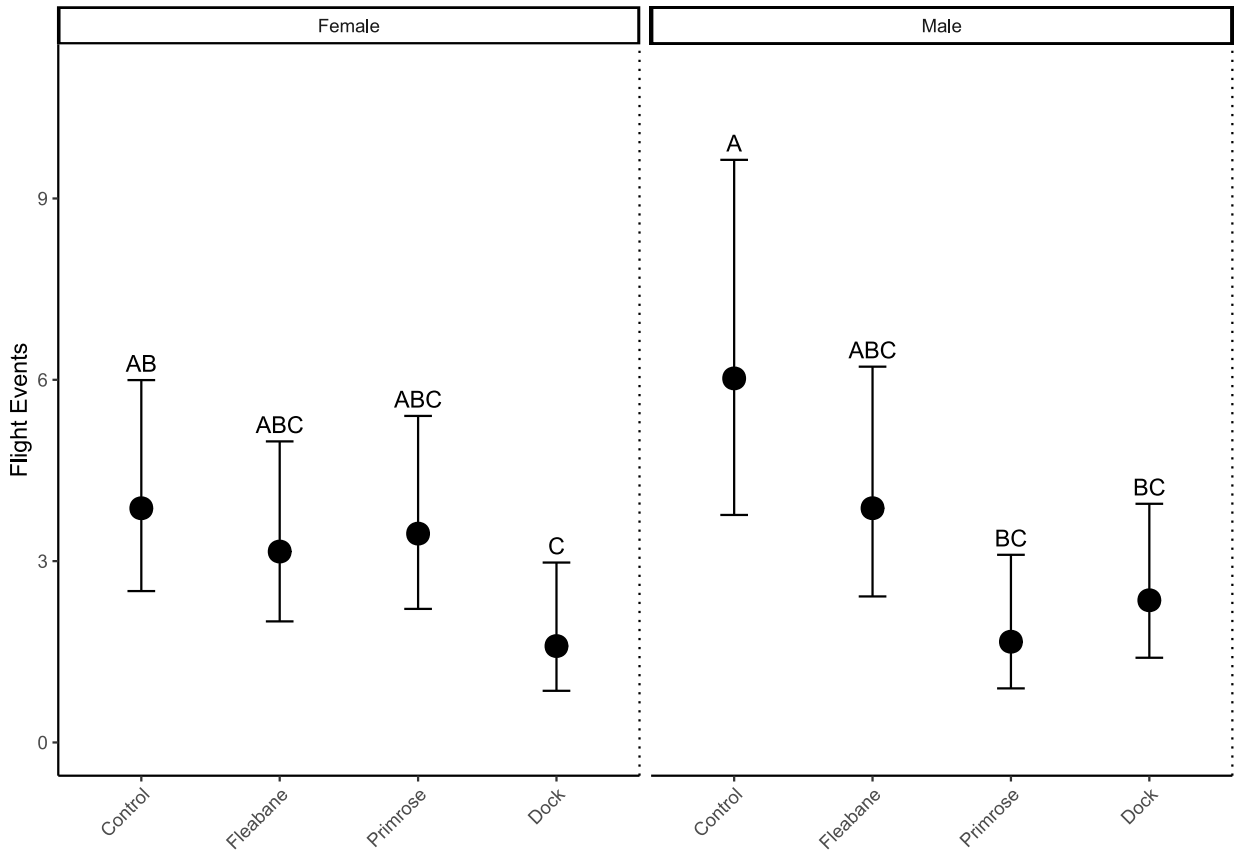

Figure 4.2. Estimated marginal means (± 95 % CI) flight events by sex of *L. lineolaris* adults taken from different weed hosts (X-axis) and subjected to both 8 and 22 h flight mill sessions. Non-fliers were omitted from all analyses. Bars sharing the same letters are not significantly different ($\alpha = 0.05$).

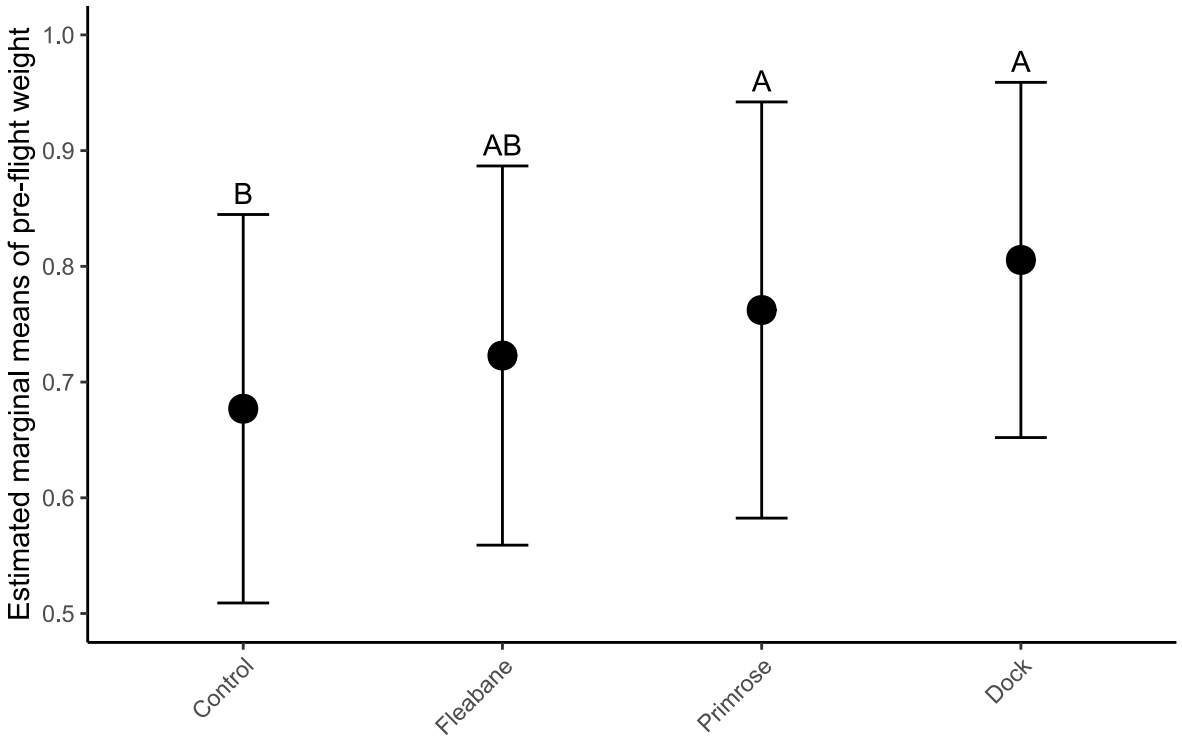

Figure 4.3. Estimated marginal means (± 95% CI) of pre-flight body-weight of *L. lineolaris* adults taken from different weed hosts (X-axis) and subjected to 22 h flight mill session. Non-fliers omitted from all weight analyses. Bars sharing the same letters are not significantly different ($\alpha = 0.05$).

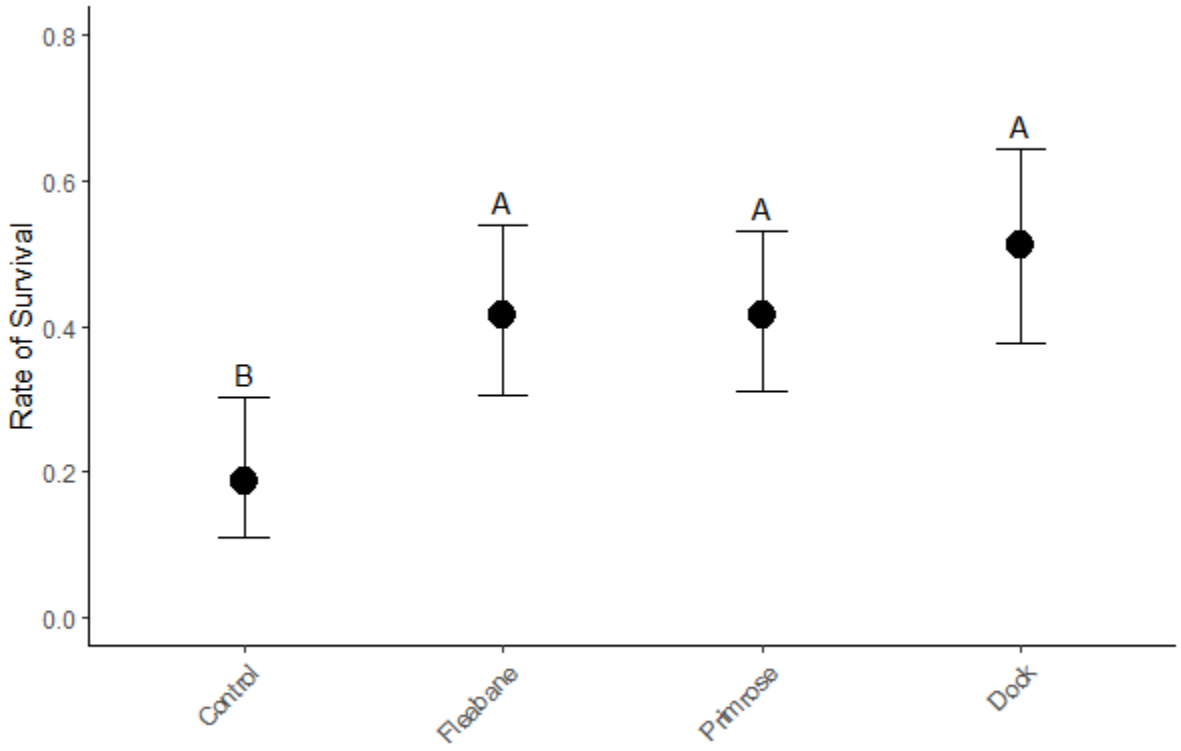

Figure 4.4. Estimated marginal means (± 95% CI) of flight mill survival of *L. lineolaris* adults taken from different weed hosts (X-axis) and subjected to 22 h flight mill session. Non-fliers omitted from all weight analyses. Bars sharing the same letters are not significantly different (α = 0.05).

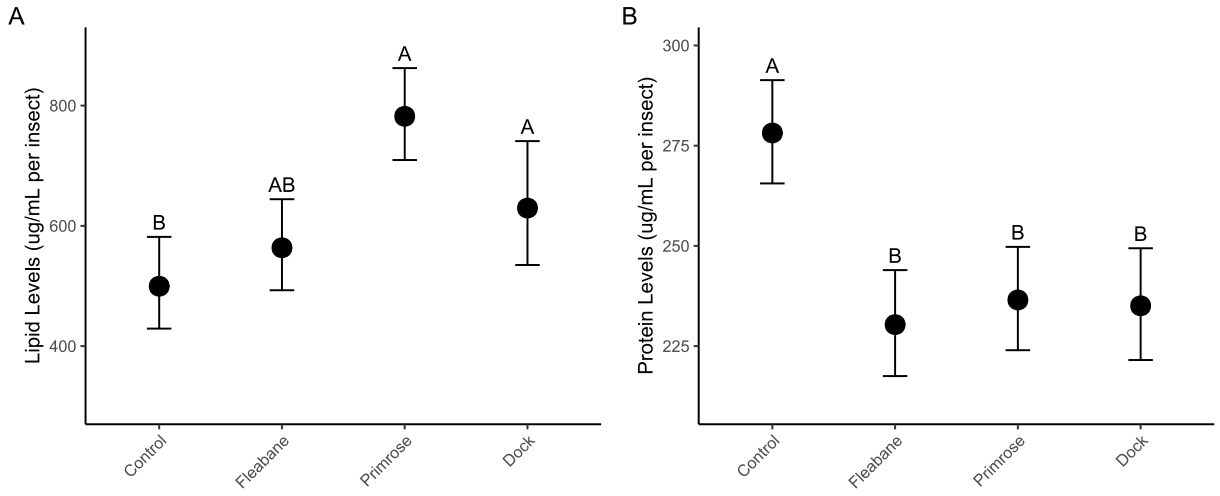

Figure 4.5. Estimated marginal means (± 95% CI) of (A) lipid, and (B) protein concentrations of *L. lineolaris* after being taken off weed hosts (X-axis). Bars sharing the same letters are not significantly different ($\alpha = 0.05$).

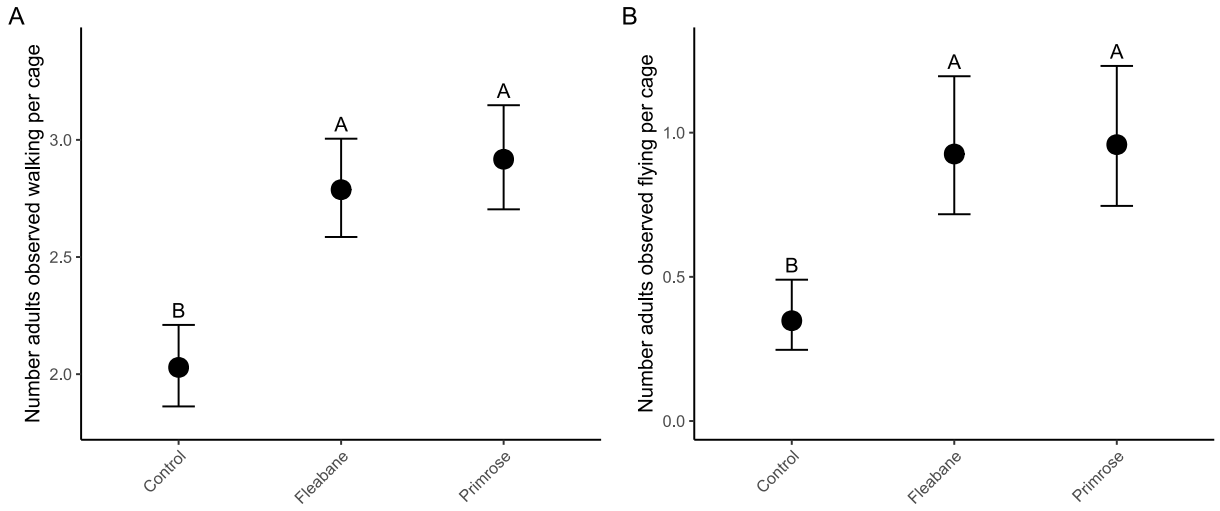

Figure 4.6. Estimated marginal means (± 95% CI) of proportion of *L. lineolaris* (A) walking, and (B) flying, after populations were taken from weed hosts and immediately placed in a mesh cage with a cotton plant. Bars sharing the same letters are not significantly different (α = 0.05).

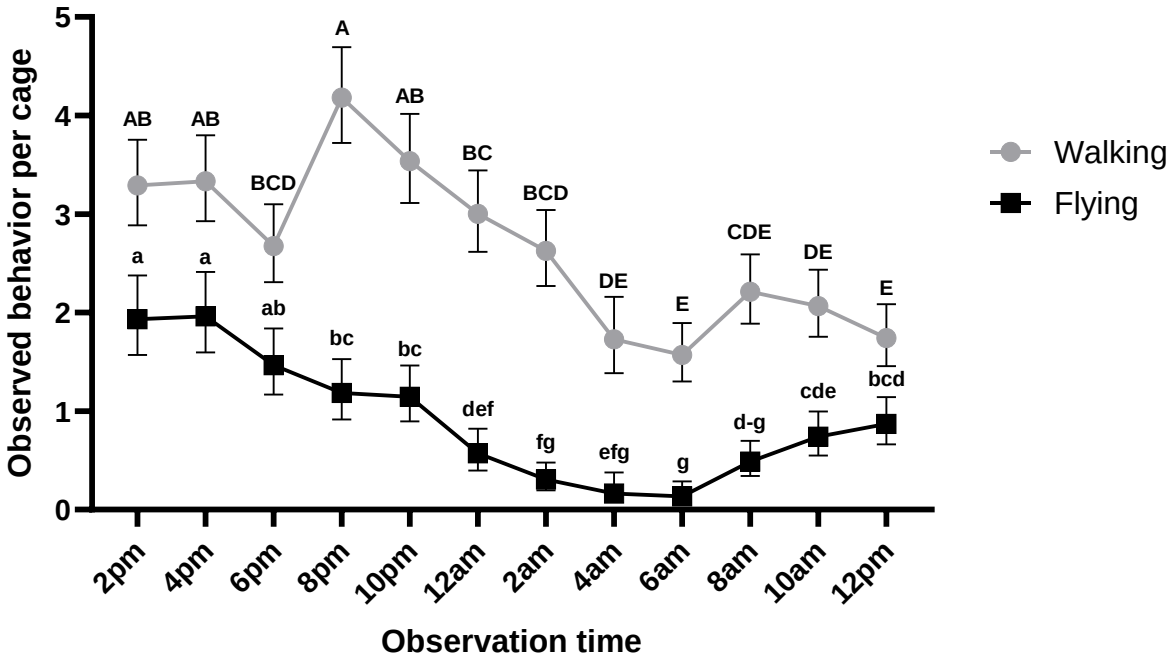

Figure 4.7. Estimated marginal means (± 95% CI) of walking and flying adults of *L. lineolaris* within mesh cages over a 24-h observation period (X-axis). Bars of similar data type and sharing the same letters are not significantly different ($\alpha = 0.05$).

Chapter 5

Insecticide treatments affect pest and natural enemy abundance in Mid-Atlantic cotton fields

Abstract

Rising pest pressure from *Lygus lineolaris* (Palisot de Beauvois) (Hemiptera: Miridae) in mid-Atlantic US cotton has necessitated an increased use of broadcast applications of insecticides. It is not known if, and how, this affects other pests and beneficial insects within the system. In a two-year study, different insecticides and insecticide rotations labeled for *L. lineolaris* control were sprayed at recommended action thresholds and measured pest and beneficial insect populations, plant injury, and yield. Insecticide treatments included acephate; acephate and novaluron; sulfoxaflor; and a thiamethoxam with acephate rotated with sulfoxaflor. All insecticides were effective at suppressing *L. lineolaris* populations below spray thresholds. There were treatment differences in how many spray applications were needed in between years and locations. Secondary pest populations, such as two-spotted spider mites (*Tetranychus urticae*), were elevated with acephate, and acephate with novaluron treatments. Plots treated with acephate and acephate with novaluron also reduced natural enemy abundance, including *Coccinellidae*, *Orius* spp., *Geocoris* spp., Neuropteran, and spiders. However, acephate and acephate with novaluron also yielded more lint per acre compared to the control. Results demonstrate that insecticide choice and rotational strategies influence natural enemy abundance and insecticides that reduce natural enemies are still capable of producing elevated cotton lint yields.

Keywords: plant bug, *Gossypium hirsutum*, biological control, insect resistance management

Introduction

The insect pest complex in mid-Atlantic US cotton has shifted in response to several key events, including boll weevil eradication (Smith 1998) and the introduction of cotton that expresses *Bacillus thuringiensis* (*Bt*) toxins targeting Lepidopteran species (Naranjo 2011). These changes resulted in a substantial decrease in broad-spectrum insecticide use in many US cotton producing states over the past several decades (Carlson and Suguiyama 1983; Cattaneo et al., 2006). Broadcast applications were used most frequently for stink bugs (Hemiptera, Pentatomidae) and thrips (Thysanoptera) in the Mid-Atlantic until the 2010s when tarnished plant bug, *Lygus lineolaris* (Palisot de Beuavois), became the most frequent and economically damaging pest. By 2016, 71% and 100% of North Carolina and Virginia cotton acreage, respectively, were receiving broadcast applications for this pest (Williams 2011, Williams 2017).

Lygus lineolaris is native to North America and can feed on over 300 plant species (Young 1986). Populations shift between wild and cultivated hosts in response to host availability and abundance in the landscape (Snodgrass et al., 1984). In the Mid-Atlantic, wild hosts senesce when cotton fields are in the pre-bloom and early bloom numerous stages, resulting in dispersal into those cotton fields. Cotton at this stage provides numerous reproductive structures on the plant (e.g., square or buds, blooms, and small bolls) for *L. lineolaris* populations to feed on (Cleveland 1982, Fleischer et al. 1988). *Lygus lineolaris* feeding on flowers, squares, bolls, and young terminals, can significantly reduce yields (Hanny et al. 1977, Aghaee et al. 2019).

Insecticides are a component of integrated pest management (IPM) programs for *L. lineolaris*. Rotation between insecticide classes, and among insecticides within the same class, is possible because there are multiple labeled insecticides available that are also effective for *L. lineolaris*. Nonetheless, products vary in overall performance in terms of both their efficacy (Reisig et al. 2011, Rogers et. al. 2022), residual activity, and the presence of insecticide resistance in some populations (Dorman et al., 2020). Insecticide resistance has been documented in mid-southern US *L. lineolaris* populations to pyrethroids, organophosphates, and carbamates (Snodgrass 1996; Snodgrass et al., 2008; Snodgrass et al., 2009). Pyrethroid and organophosphate-resistant populations have been documented, potentially because these classes of insecticides were commonly used for other pests over several decades (e.g., thrips, stink bugs, bollworm; Dorman et al., 2020). Resistance to neonicotinoids, although measurable in some populations, is low likely because there is limited foliar use of neonicotinoids following planting (Dorman et al., 2020).

Novaluron, sulfoxaflor, and flonicamid are among the newest modes of action registered for *L. lineolaris* control. The insect growth regulator novaluron acts to disrupt chitin growth during molting and has sublethal effects on *L. lineolaris* adults such as reduced egg viability and fecundity (Retnakaran 1985; Catchot et al., 2021). Novaluron targets immatures and pairing with an insecticide that targets adults is often necessary. Sulfoxaflor is an insecticide in the sulfoxamines class, which acts systemically targeting sap sucking pests (Babcock et. al., 2010). Flonicamid, commonly used to target aphids, can control *L. lineolaris* which makes this application practical in areas where both pests occur above economic injury levels (Morita et al. 2014). Because of the frequency of insecticides now being sprayed, and the addition of different modes of action, it itself is important to understand how the pest and beneficial arthropod assemblage responds.

Natural enemies can be vital in reducing primary pests and preventing secondary pest outbreaks in cotton (Young 1989, Obrycki and Kring 1998, Knutson et al., 2005). There are numerous natural enemies that inhabit mid-Atlantic cotton that provide some level of pest control. Big-eyed bug (Hemiptera: *Geocoris* spp.) and assassin bug (Hemiptera: Reduviidae), for example, prey on *L. lineolaris* and bollworm, *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) (Knutson et al. 2005). Several spider species (Araneae) are also efficient predators of arthropods in cotton, capable of reducing immature and adult *L. lineolaris* numbers (Young 1989). Other generalist biological control agents are predators of two-spotted spider mites, aphids, and *H. zea* and *Pentatomidae* eggs. Minute pirate bug (Hemiptera: *Orius* spp.) are voracious feeders on *H. zea* and *Pentatomidae* eggs. Green lacewings (Neuroptera: Chrysopidae) and brown lacewing (Neuroptera:) assist in controlling aphids and *T. urticae*. Chrysopids also feed on *H. zea* and *Pentatomidae* eggs (Knutson et al., 2005). Ladybird beetles (Coleoptera: Coccinellidae), are used widely as biological control agent for aphids, and are commonly found in cotton (Obrycki and Kring 1998). Predators can work in concert with reduced-risk chemical control programs to minimize economic losses, but predator populations can be inadvertently reduced or eliminated by broad-spectrum insecticides through a combination of lethal and sublethal effects (Obrycki and Kring 1998; Ruberson et al., 1999; Elzen 2001; Tillman et al., 2000).

Insecticides targeting *L. lineolaris* may influence secondary pest outbreaks by reducing predators or, in the specific case of acephate and *T. urticae*, generate hormoligosis (Trichilo et al., 1993; James et al., 2002). Historically, *T. urticae* and aphid outbreaks are rare and sporadic in mid-Atlantic cotton and this may change with repeated sprays for *L. lineolaris* control. Other pests, like

Pentatomidae and *H. zea*, may be controlled by *L. lineolaris* applications and become less problematic (Greene et al., 2001).

The purpose of this study was to better understand how *L. lineolaris* foliar sprays affected cotton biological control agents in the mid-Atlantic, and to determine the potential benefits and costs of product selection and rotations. More broadly, our goal was to improve cotton IPM by providing producers with the information that they need to minimize applications, human health risks and environmental residues from insecticides.

Methods

Field experiments

Experiments were conducted at the Tidewater Agricultural Research and Extension Center in Suffolk, Virginia, and at the Vernon James Research and Extension Center in Plymouth, North Carolina in 2020, and 2021. Plots were planted with DP 1646 B2XF Elite seed (Bayer CropScience, St. Louis, MO). Treatments were arranged in a randomized complete block design with four replicates. In 2020, treatments included: 1) an untreated control; 2) acephate at 0.55 kg ai/ha, (Orthene, AMVAC, Newport Beach, CA); 3) acephate at 0.55 kg ai/ha and novaluron 0.08 kg ai/ha (Diamond, ADAMA, Raleigh, NC); 4) sulfoxaflor at 0.08 kg ai/ha, (Transform WG, Corteva Agriscience, Indianapolis, IN); 5) flonicamid at 0.17 kg ai/ha (Carbine 50 WG, FMC Corporation, Philadephia, PA); and 6) a rotation of thiamethoxam (0.072 kg ai/ha, Centric, Syngenta Crop Protection, Greensboro, NC), with acephate, and sulfoxaflor (rotational treatment). In 2021, we did not include flonicamid or thiamethoxam treatments. Thiamethoxam was not

included in rotation because the first spray was made following bloom and NC Cooperative Extension recommends not using neonicotinoids (e.g., thiamethoxam) past June (Reisig and Huseth, 2022). Flonicamid was dropped from the analysis since there were not enough replicates where it was included. All treatments ceased when cotton plants were less than 5 nodes above white flower.

In Virginia, plots were 4.9 m wide (8 rows) by 10.7 m long in 2020, and 14.6 m wide (24 rows) by 25.9 m long in 2021. Replicates were separated by 7.31 m bare alleys and plots were separated by 2.13 m cutouts where cotton was removed. Virginia plots were planted on 2 June 2020 and 12 May 2021. In North Carolina during 2020, plots were 10.97 m wide (12 rows) by 12.19 m long. In 2021, plots were 14.63 m wide (16 rows) by 15.24 m long. Plots and replicates were separated by 2.13 m alleys. North Carolina cotton was planted on 1 June 2020 and 16 June 2021. Treatments were applied when *L. lineolaris* populations reached Cooperative Extension recommended thresholds (8 per 100 sweeps pre-bloom and 2.5 per beat cloth post-bloom). Treatments were applied using a Spider Spray Trac sprayer calibrated to deliver 186 liters/ha through 8002TJ nozzles at 262 kPa in Virginia and a John Deere 6000 Sprayer calibrated to deliver 117 liters/ha using two TeeJet TX-6 nozzles per row at 379 kPa in North Carolina.

Experimental fields were maintained, including the use of mepiquat chloride and herbicides, as needed, using standard agronomic practices for both states. Other than insecticide treatments for *L. lineolaris*, the only insecticides applied were sprayed directly in the seed furrow during planting for thrips control (imidacloprid at 0.64 kg ai/ha, Admire Pro, Bayer Crop Science LP, St. Louis, MO). In Virginia, cotton was machine harvested with a two-row John Deere 9930 mechanical picker on 18 November 2020 and 9 November 2021. In North Carolina, cotton was

machine harvested in the middle two rows with a two-row John Deere 9920 mechanical picker on 28 October 2020 and 20 October 2021. In Virginia, cotton lint was separated during the ginning process and lint weight was taken after ginning. Lint yields in North Carolina were calculated from the seed weight assuming 40% gin turnout.

Insect Sampling

Sweep nets and beat cloths were used to measure pest and beneficial artrhopod species on a weekly basis from the first week of squaring until cotton plants were less than 5 nodes above white flower in Virginia. Sweep nets were used from squaring until the first week of bloom and beat cloths were used following the first week of bloom in North Carolina. Sweep nets and beat cloths more accurately capture adult and immature *L. lineolaris* populations, respectively (Musser et al. 2007). Twenty-five sweep samples and or the average of three beat cloth samples were taken in each plot. Thresholds were eight per 100 sweeps and less than 80% square retention pre-bloom and 2.5 or greater bugs per drop post-bloom.

In Virginia, sweep nets were used to monitor populations of *Pentatomidae* and seven natural enemies (coccinellid larvae and adults, *Reduviidae* spp., *Orius* spp., *Geocoris* spp., Chrysopidae and Hemerobiidae. larvae and adults, and spiders). *Pentatomidae* and beneficial insects were identified to family and spiders were identified to order. *Pentatomidae*. counts were not collected in Virginia in 2020 and no *Pentatomidae* spp. or beneficial insect data was collected in North Carolina. Sampling for pests and beneficial insects in Virginia was conducted on four dates in 2020 (28 Jul, 17 Aug, 24 Aug, and 2 Sept) and weekly following the first spray application in 2021. In North Carolina during 2021, *Pentatomidae* sampling was performed weekly alongside

L. lineolaris sampling. *Pentatomidae* spp. and beneficial insects were sampled using 25 sweeps performed in rows that were not used for *L. lineolaris* samples or yield.

In Virginia, aphids and *T. urticae* were sampled following spray applications (28 Jul, 17 Aug, 24 Aug, and 2 Sept, in 2020; and 2 Aug and 19 Aug in 2021). Sampling in North Carolina was performed once on 11 Aug in 2020. Sampling involved removing 1 of the 10 uppermost expanded leaves from ten random cotton plants in each plot. Leaves were washed in soapy water to remove mites and insects. Vacuum filtration was used to filter samples and mites and aphids were counted and identified using dissection microscopes.

Square retention and internal boll injury

Square retention and internal boll injury provide indirect information regarding *L. lineolaris* presence (Musser et al., 2007, Aghaee 2019) and indicate when a threshold is reached for *Pentatomidae* in cotton (Greene et al., 2001). Square retention and boll injury were measured at each location. Square retention was measured by recording the presence or absence of squares on the top three positions below the terminal on five random plants in each plot. In 2020, square retention was measured on 24 Jul, 28 Jul, and 3 Aug in Virginia, and 29 Jul and 5 Aug in North Carolina. Data for all four replications were combined to calculate retention for each treatment. Internal boll injury was measured by collecting 25 random 2.25 cm diameter bolls per plot. Bolls were cracked open to inspect for the presence of *H. zea* larvae or *H. zea* feeding, and internal injury from *Pentatomidae* or *Lygus* spp. feeding (i.e., warts, stained lint, punctures on the internal carpal wall). In Virginia, boll sampling was performed on 10, 17, and 24 Aug in 2020, and on 5 and 11 Aug in 2021. In North Carolina, boll sampling was performed on 11 Aug in 2020.

Data analysis

Data were analyzed in R (Version 4.3.1 R Core Team, 2023) using the lme4 (Bates et. al., 2015), glmmTMB (Brooks et. al., 2017), emmeans (Lenth, 2018), and multcomp (Hothorm et. al. 2023) packages. Sweep net data was used for analyzing *L. lineolaris* adult populations and beat cloth data was used for analyzing *L. lineolaris* nymph populations. Data fitted to a Poisson distribution included numbers of *L. lineolaris* nymphs, *L. lineolaris* adults, *Pentatomidae, T. urticae*, square retention, boll injury, and natural enemies. Categories of natural enemies modeled were Coccinelids, *Reduviidae*, *Geocoris* spp., *Orius* spp., hemipterans, neuropterans, spiders, and all predators. These data categories were subjected to a generalized linear mixed effect model (GLMM) using lme4 with the model selection determined by Akaike information criterion (AIC). Aphid, *T. urticae* and *Pentatomidae* data were fitted to a zero-inflated Poisson GLMM using glmmTMB with the model determined by AIC. In all analyses, insecticide treatment, experimental block, and site year were fixed effects with week of year sampled as a random effect. Lint yield data were fitted to a Gaussian distribution and subjected to a GLMM with insecticide treatment and site year as fixed effects. The results of all GLMM were subjected to a Tukey's HSD post-hoc test using the emmeans package. Compact letter displays for all post-hoc tests were determined by multcomp.

Results

Lygus Populations and Outcomes of Applications

Initial insecticide applications were triggered when *L. lineolaris* field populations reached economic threshold levels. In Virginia, adult populations reached threshold in July both years with treatments applied on 21 Jul 2020 and 20 Jul 2021 (**Table 5.1.**). Nymph populations reached threshold again mid-August in both years, which required additional treatments. In North Carolina, adult populations reached threshold in July both years with treatments applied on 22 Jul 2020 and 16 Jul 2021. In 2020, nymph populations did not reach threshold after the initial treatment. In 2021, *L. lineolaris* populations peaked after the initial treatment with all plots requiring an additional treatment in late-July to early-August.

Populations of *L. lineolaris* were measured in both locations and years following squaring and prior to the initial treatment and ending in late August (**Table 5.2**). Higher adult densities occurred in early summer and declined later in the growing season. Populations in the control plots were 2.30 times higher per plot than acephate-treated plots (**Figure 5.1A.**), and 2.12 times higher than the levels in acephate with novaluron-treated plots. The control plots had 2.21 times higher adult densities compared to sulfoxaflor treated plots, while adults in the rotational treatment were lowered by 1.51 times per plot compared to the control. No treated treatments were statistically different from each other.

Nymph populations increased after the initial infestation in mid-late July in all years and locations. The control maintained a nymph density of 2.11 times the level in acephate treated plots (**Figure 5.1B.**), while acephate with novaluron reduced nymphs at a rate of 2.57 per plot. Sulfoxaflor reduced nymph densities from the control by 2.53 times per plot. Nymph levels in the

rotational treatment were reduced by 2.52 times per plot compared to the control. All treatments were similar to each other in their control of nymphs.

Other Pest and Beneficial Insect Populations

Additional pests were present within plots with some being populations varying between treatments in certain years (**Table 5.3.**). A single adult *H. zea* was sweep net sampled in Virginia in 2021 and no larvae or larval feeding were observed in other years or locations. *Pentatomidae* were observed in low levels with 12 adults and no nymphs sampled across the entire study. Models on *Pentatomidae* populations were unable to converge due to low numbers. Aphids were also present in low levels across the study. The control plots had aphid levels at 0.81 times the acephate treated plots, 0.61 times acephate with novaluron treated plots, 1.20 times sulfoxaflor treated plots, and 0.85 times the rotational treatment treated plots (**Figure 5.2A.**). Densities in acephate with novaluron treated plots were higher than sulfoxaflor treated plots by 1.95 times. All other treatments had similar aphid populations.

Tetranychus urticae were observed across the study in Virginia, with 98.8% of *T. urticae* sampled on a single date following the initial treatment in Virginia 2020. The control treated plots had lower density of *T. urticae* compared to acephate treated plots by 0.46 (**Figure 5.2B.**), acephate with novaluron treated plots by 0.15, sulfoxaflor treated plots by 0.23, and the rotational treated plots by 0.24. *Tetranychus urticae* populations were further elevated in all treatments except acephate, with acephate treated plot populations at 0.46 times that of acephate with novaluron treated plots, 0.51 times that of sulfoxaflor treated plots, and 0.53 times that of the rotational treated plots. Acephate with novaluron treated plot densities were elevated compared to those in

sulfoxaflor treated plots and rotational treated plots by 1.52 and 1.59 times respectively. Sulfoxaflor and the rotational treated plots experience similar *T. urticae* levels with a ratio of 1.04 between them (95%-CI: 0.81 to 1.33; z = 0.47, p = 0.9900).

Natural enemies were measured exclusively in Virginia. A total of 283 beneficial insects and spiders were collected during sampling. Coccinellidae were the dominant group of beneficial arthropods making up 38% of the specimens sampled. Other beneficial arthropod groups were hemipterans (24%), neuropterans (21%), and spiders (17%). Coccinellids and neuropterans did not significantly vary between treatments. Spider levels did not have significance between treatments despite almost no spiders seen in plots treated with acephate. Predatory hemipterans had higher densities in control plots by 4.92 times of acephate treated plots, 2.10 times acephate with novaluron treated plots, 1.9 times sulfoxaflor treated plots, and 1.78 times rotational treated plots (**Figure 5.2C.**). Predatory hemipteran densities in acephate treated plots were lower than those of acephate with novaluron by 0.43 times, sulfoxaflor by 0.39 times, the rotational by 0.36 times. No other treatments were significantly different from each other. Total predators were elevated in control plots by 1.76 times plots treated with acephate, 1.76 times plots treated with acephate with novaluron, 1.44 times plots treated with sulfoxaflor, and 1.12 times plots treated with the rotation (**Figure 5.2D.**). Predator levels between plots treated with acephate and acephate with novaluron were the same. Acephate and acephate with novaluron total predator levels were both below sulfoxaflor treated plots by a factor of 0.82, and the rotational treated plots by a factor of 0.64. Sulfoxaflor treated plots had total predator densities lower by 0.78 times those seen in the rotational treatment.

Plant Injury

Square retention was measured in 2020. Control plots averaged 94.4% square retention across both locations and sampling dates. The control had a higher square retention than the 93.7% observed in acephate treated plots with a ratio of 1.01, and the 92.5% retention in the rotational treated plots with a ratio of 1.02. The control exhibited a lower square retention than the 96.8% observed in acephate with novaluron treated with a ratio of 0.98, and 98.2 % in sulfoxaflor treated plots with a ratio of 0.96. Sulfoxaflor treated plots had higher retention than rotational treated plots by 1.06 times. No other treatments had significance between square retentions. Location had an impact on square retention with North Carolina exhibiting retention 1.08 times higher than in Virginia.

Boll injury was sampled in North Carolina in 2020 and Virginia in 2020 and 2021. No bolls were injured by *H. ze*a; all injury was caused by feeding hemipterans such as *L. lineolaris* and *Pentatomidae* spp. Injured bolls were distinguished by warts and or discoloration of internal lint. Boll injury was not significantly different across treatments.

Yield

Insecticide treatments had significantly different lint yields (χ^2 = 9.52, df = 4, P = 0.0494). The control (1056 ± 70 kg/ha) had lower lint yields than the 1310 ± 47 kg/ha produced by acephate treated plots (**Figure 5.3.**), the 1296 ± 33 kg/ha produced in acephate with novaluron treated plots, but similar lint yields to the 1229 ± 56 kg/ha produced in sulfoxaflor treated plots, and the 1201 ± 53 kg/ha produced in the rotational plots. The relationships between plots treated with insecticides did not differ significantly.

Discussion

This study demonstrated the efficacy of insecticides to manage *L. lineolaris*, while also highlighting impacts on natural enemies and secondary pests. All insecticides reduced populations of immature *L. lineolaris*, but the rotational treatment was slightly less effective on adults. In general, the acephate and novaluron treatment had the most unfavorable profile for flaring secondary pests, averaging the most aphids and spider mites relative to other treatments. Acephate had the greatest negative effect on spider populations and significantly reduced total natural enemy numbers relative to the control. However, both treatments that included acephate also significantly increased yield relative to the control. Sulfoxaflor had a favorable profile for total natural enemy populations, while reducing aphid levels, but significantly flared spider mites relative to the control. These sulfoxaflor results appear to be unique as the insecticide is considered to be safe to use in cotton fields containing spider mites (Brown et. al., 2015). It should be noted that our study was limited to North Carolina and Virginia in 2020 and 2021 and does not provide enough evidence to support a broad statement on this phenomenon. These results also suggest that all the insecticides tested can have negative impacts on natural enemies or secondary pests. Insecticide recommendations for *L. lineolaris* in Mid-Atlantic cotton are complicated by the pressure to improve yield while preserving beneficial insects, and avoiding increases of secondary pests.

Virginia consistently had higher pressure from *L. lineolaris* throughout the study in both years compared to North Carolina. The rotational treatment was the only treatment that was unsuccessful in lowering adult populations compared to the control. Nonetheless, this treatment

required the same number of foliar sprays as the other treatments. Furthermore, while acephate alone lowered adult and nymph populations relative to the control, an additional treatment was needed in Virginia due to the population of nymphs increasing in those plots within six days after the application (18 August 2020). All other treatments had the same number of applications during the same year at their respective location. This may be an additional indication that acephate is losing efficacy against *L. lineolaris* in mid-Atlantic cotton (Dorman et al 2020).

Pest populations other than *L. lineolaris* remained low throughout the study with a single outbreak from *T. urticae*. *Tetranychus urticae* populations had a drastic increase on 29 July 2020 in Virginia following drought conditions (English-Loeb 1990). All treated plots had higher populations *T. urticae* relative to the control. Outbreaks of this nature are most commonly caused by non-target insecticide treatments, such as organophosphates, acting as a physiological stimulant to *T. urticae*, increasing fecundity, while novaluron treatments can also result in increased populations (Leigh 1980, Martinez-Rocha et. al., 2008). Insecticide impacts on natural enemy populations may have also contributed to the increase in *T. urticae* population (Bartlet et. al. 1968, Campbell 1973, Trichilo et. al. 1993, James et al., 2002). The presence of *Pentatomidae* was consistently low in all treatment and control plots, and their population levels as a secondary pest may have prevented the detection of a difference made by the treatments if there were any. The *Pentatomidae* are a significant pest in the mid-Atlantic (Cook and Threat 2021, Cook and Threat 2022), but pesticide response from this pest was not captured due to low populations in this study. Aphids populations were relatively low in all plots over both years. Removal of beneficial insects in plots treated with acephate may have contributed to slightly elevated populations compared to

plots treated with sulfoxaflor, while novaluron has been demonstrated to increase aphid species when biocontrol tactics are disrupted (Beers et. al., 2016).

The beneficial insects that were sampled in this study did not substantially contribute to the natural enemy abundance in cotton due to their low populations. Only 10 total *Geocoris* spp. and 6 *Reduviidae* were observed throughout the study, making up 15% and 9% of total predatory hemipterans, respectively. In contrast, *Orius* spp. contributed to 76% of predatory hemipterans. Lacewing larvae made up 12% of all lacewings and coccinellid larvae made up 17% of all coccinellids caught during the study. This could indicate issues in the bias of collection method, as sweep nets best captured the top canopy of a cotton plant while larvae may exist in higher populations lower down on the cotton plants.

Adult coccinellids, spiders (Arameae), and *Orius* spp. were the most prevalent beneficial insects across years and locations. Adult coccinellids accounted for 32% of all-natural enemies captured and the population was persistent throughout the study. Spiders were common in plots and were observed feeding on *L. lineolaris* adults and nymphs during sampling. *Orius* spp. were almost exclusively seen in 2021, making up 18% of the natural enemy abundance. Their populations may have been higher as the insect's small size could have allowed them to exist in denser populations in the uppermost portion of the cotton plant, underestimated due to sampling bias by sweep nets. Although there was no clear correlation between *Orius* spp. and pest populations, *Orius* spp. could be important in preventing aphid and *T. urticae* population outbreaks.

Acephate and acephate with novaluron lowered natural enemy populations in cotton plots. Negative impacts of acephate on spider and predatory hemipterans were expected (Theiling and

Croft, 1988, Maienfisch et al. 2001). The reduction of natural enemies may have accounted for the additional treatment used in Virginia plots during 2020. Decreases in acephate efficacy on *L. lineolaris* populations has been documented in this region (Dorman et. al., 2020). Acephate with novaluron did not lower any single class of natural enemy, however, natural enemy levels were lowered across all categories. Insect growth regulators are usually less detrimental on natural enemies and it is likely the inclusion of acephate responsible is responsible for the effect seen in our study (Naranjo et. al., 2003, Naranjo et. al., 2004, Beuzelin et. al., 2010). Sulfoxaflor selectively targets sap feeding insects and is avoided by insect predators (Babcock et al. 2010, Zhu et al. 2011). Natural enemies in the rotational treatment populations remained consistent with the control, indicating that when acephate is not used exclusively, natural enemies have adequate opportunity to recover in population. Rotating acephate with sulfoximines and neonicotinoids, can allow its inclusion in cotton IPM programs.

In this study, treatment did not have an impact on both direct (internal boll injury), or indirect (square retention) plant injury. In past studies, square retention and boll injury have been shown to be significantly decreased by insecticide treatments, with boll injury having higher variation among treatments (Dorman et. al. 2020). Square retention issues can occur due to stresses beyond insect injury, such as water, heat stressors, and nutrient deficiencies (Guinn, 1982), but are uncommon due to environment in the Mid-Atlantic system. The number of bolls injured can be influenced by the amount of adult *L. lineolaris*, rather than overall size of the population, which may not be calculated for due to sampling method bias (Greene et. al. 1999). However, it was not distinguished between injury levels between bolls, which may have masked potential differences.

No significant differences existed among locations and years, which indicates that additional stressors or discrepancies among life stage did not have an effect on plant injury results.

Acephate and acephate with novaluron were the only treatments that increased lint yield from the control. These treatments were effective despite natural enemy loss. Similarly, sulfoxaflor and rotational treatments have increased yields in other studies (Seibert et al 2012, Gore et. al., 2014, Dorman et. al., 2020). Differences in yield are often more apparent in years with higher pest pressure. Notably, North Carolina plots exhibited low *L. lineolaris* levels during both years, despite exceeding the threshold multiple times.

Insecticide treatments are needed to control *L. lineolaris* in mid-Atlantic cotton. Aphids and *T. urticae* can be periodically elevated and increased with the use of certain insecticides. Although the overall risk of secondary pests was low in our studies, caution is advised if broad-spectrum insecticides are used across the landscape in varying environmental conditions. This study did not monitor the effect of predation by natural enemies or the predation capacity by certain natural enemy groups. Rotation of insecticides remains a recommended tool for slowing the evolution of resistance to specific classes.

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Table 5.1. Dates of Insecticide Application in Virginia and North Carolina over 2020 and 2021 as Dictated by *L. lineolaris* Population Levels Reaching Economic Thresholds

Treatment	Rate	2020 Application Date		2021 Application Date	
		Virginia	North Carolina	Virginia	North Carolina
Acephate	0.59 L /ha	21 Jul, 18 & 26 Aug	22 Jul	20 Jul, 9 Aug	16 Jul, 8 Aug
Acephate with Novaluron	0.59 L/ha 0.44 L/ha	21 Jul, 26 Aug 21 Jul	22 Jul	20 Jul, 9 Aug	16 & 23 Jul
Sulfoxaflor	1.64 L/ha	21 Jul, 18 Aug	22 Jul	20 Jul, 9 Aug	16 Jul, 13 Aug
Thiamethoxam/ Acephate rotated with Sulfoxaflor	1.64L/ha 0.59 L/ha 1.64 L/ha	21 Jul 21 Jul 11 Aug	22 Jul 22 Jul -	- 20 Jul 9 Aug	- 16 Jul 23 Jul

Table 5.2. Summary Statistics for *Lygus lineolaris*

Variable	Fixed effect	df	χ^2	P
Adult				
(sweep net)	Treatment	4	19.46	0.000***
	Replicate	3	9.91	0.019*
	Site Year	2	27.10	0.000***
Nymph				
(beat cloth)	Treatment	4	221.78	0.000***
	Replicate	3	15.00	0.002*
	Site Year	3	240.01	0.000***

Asterisks represent significant significances (*P<0.05, ***P<0.001)

Table 5.3. GLMM Summary Statistics for Secondary Pests and Natural Enemies

Variables	Fixed effects	df	χ^2	P
Pentatomidae[1]				
	Treatment	4	0.661	0.9561
	Block	-	-	-
	Site Year	-	-	-
Aphididae[1]				
	Treatment	4	18.70	0.0009***
	Block	3	2.12	0.5481
	Site Year	2	18.23	0.0001***
Tetranychus urticae[1]				
	Treatment	4	259.66	0.0000***
	Block	-	-	-
	Site Year	2	1.48	0.4774
Coccinellidae[2]				
	Treatment	4	6.88	0.1425
	Block	3	4.03	0.2582
	Site Year	1	2.03	0.1545
Neuroptera[2]				
	Treatment	4	2.54	0.6376
	Block	3	4.45	0.2171
	Site Year	-	-	-
Arachnida[2]				
	Treatment	4	9.13	0.0570
	Block	3	1.19	0.7558
	Site Year	1	3.32	0.0683
Hemiptera[2]				
(Predatory)	Treatment	4	13.35	0.0097*
	Block	3	5.02	0.1703
	Site Year	1	14.61	0.0001***
All Natural Enemies[2]				
	Treatment	4	15.56	0.0037*
	Block	3	6.78	0.0791
	Site Year	1	0.19	0.6650

Asterisks represent significant significances (*P<0.05, ***P<0.001)

[1]Designantes pests, [2]designates natural enemies

Table 5.4. GLMM Summary Statistic for Plant Injury

Variables	Fixed effects	df	χ^2	P
Square Retention				
	Treatment	4	13.35	0.0097***
	Block	-	-	-
	Site	1	27.83	0.0000***
Boll Injury				
	Treatment	2	6.65	0.1554
	Block	-	-	-
	Site Year	1	1.30	0.4929

Asterisks represent significant significances (*P<0.05, ***P<0.001)

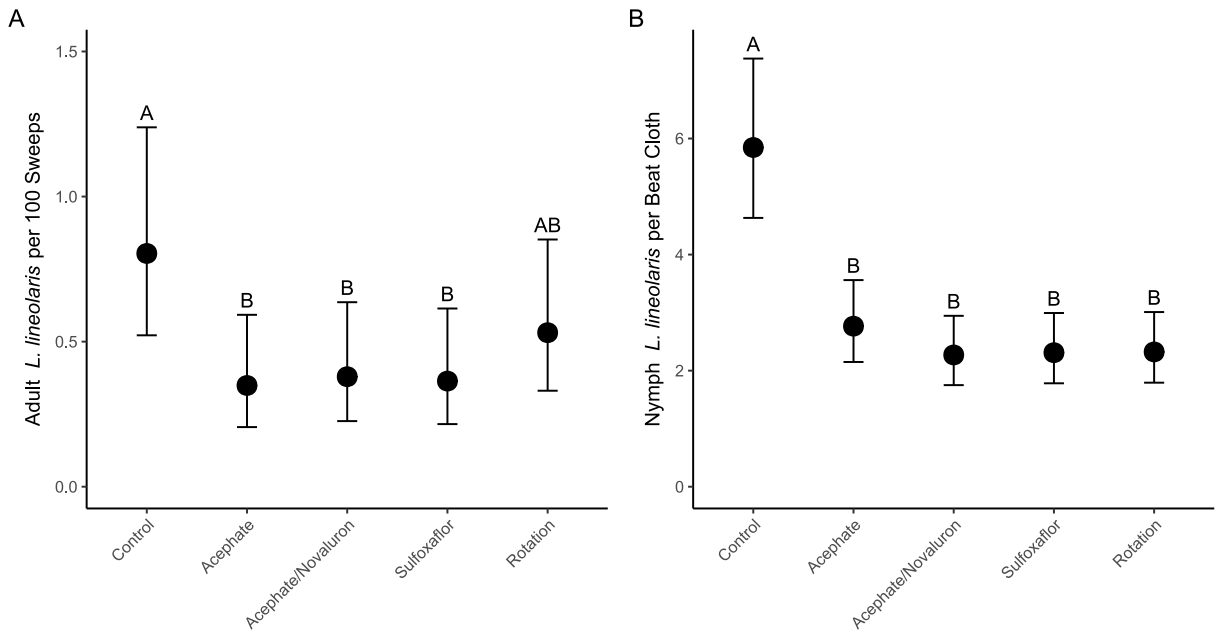

Figure 5.1 Estimated marginal mean (± 95% CI) of *L. lineolaris* populations of **A)** nymphs caught via beat cloths post bloom and **B)** adults caught via sweep nets beginning after the first treatment through August of 2020 and 2022. Treatments were actively applied throughout sampling period when pest threshold was reached. Mid-Atlantic thresholds for sweep nets are eight *L. lineolaris* per 100 sweeps and for beat cloths are 2.5 *L. lineolaris* per 1.5 row-m.

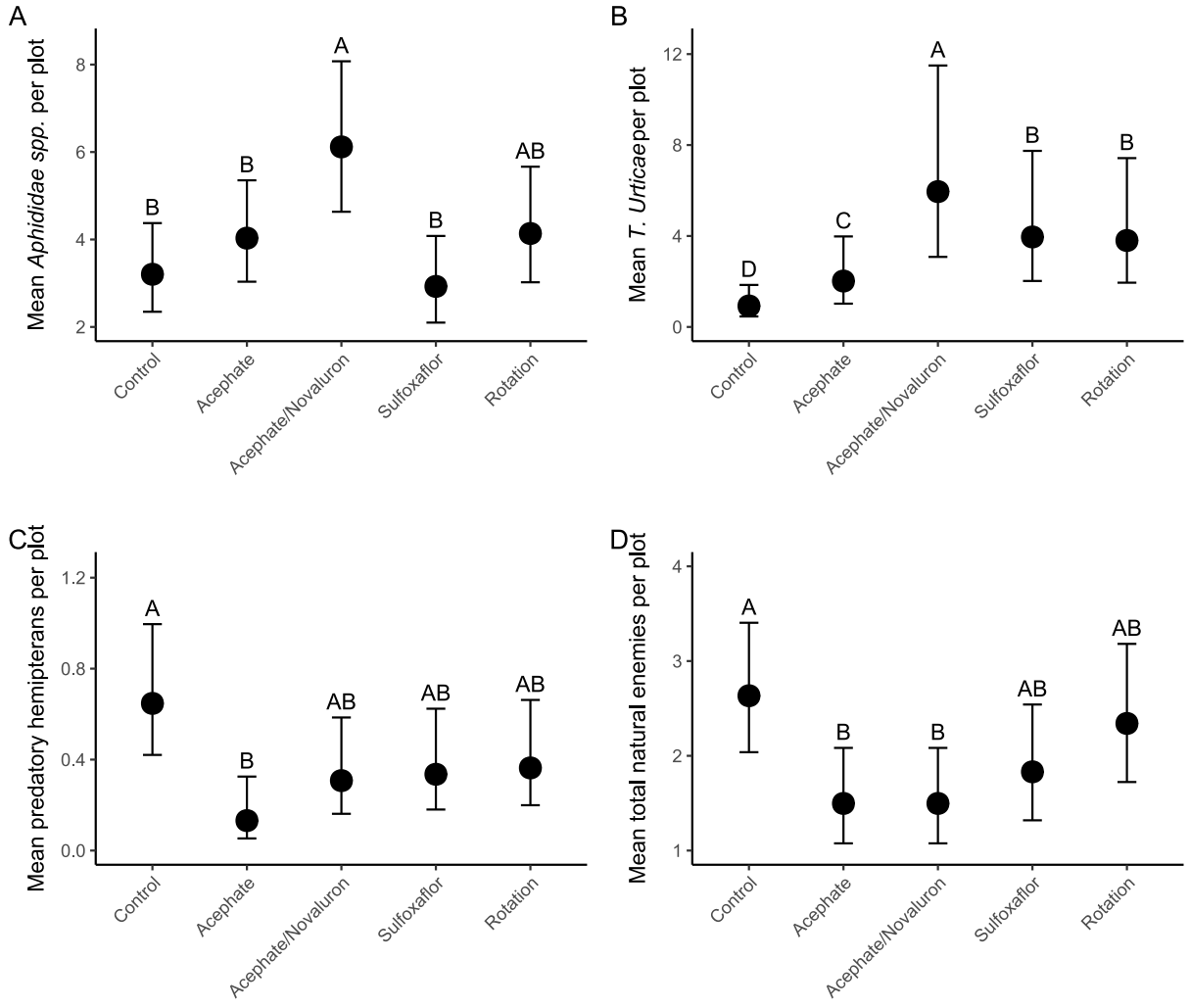

Figure 5.2. Estimated marginal mean (± 95% CI) insect abundance of secondary pests and natural enemies between treatments. Secondary pests **A)** aphid spp. and **B)** *Tetranychus urticae* were measured by removal of one uppermost expanded leaf from ten random cotton plants per plot and counted via a microscope. Natural enemies consisting of **C)** predatory hemipterans and **D)** and total natural enemies were measured via sweep nets.

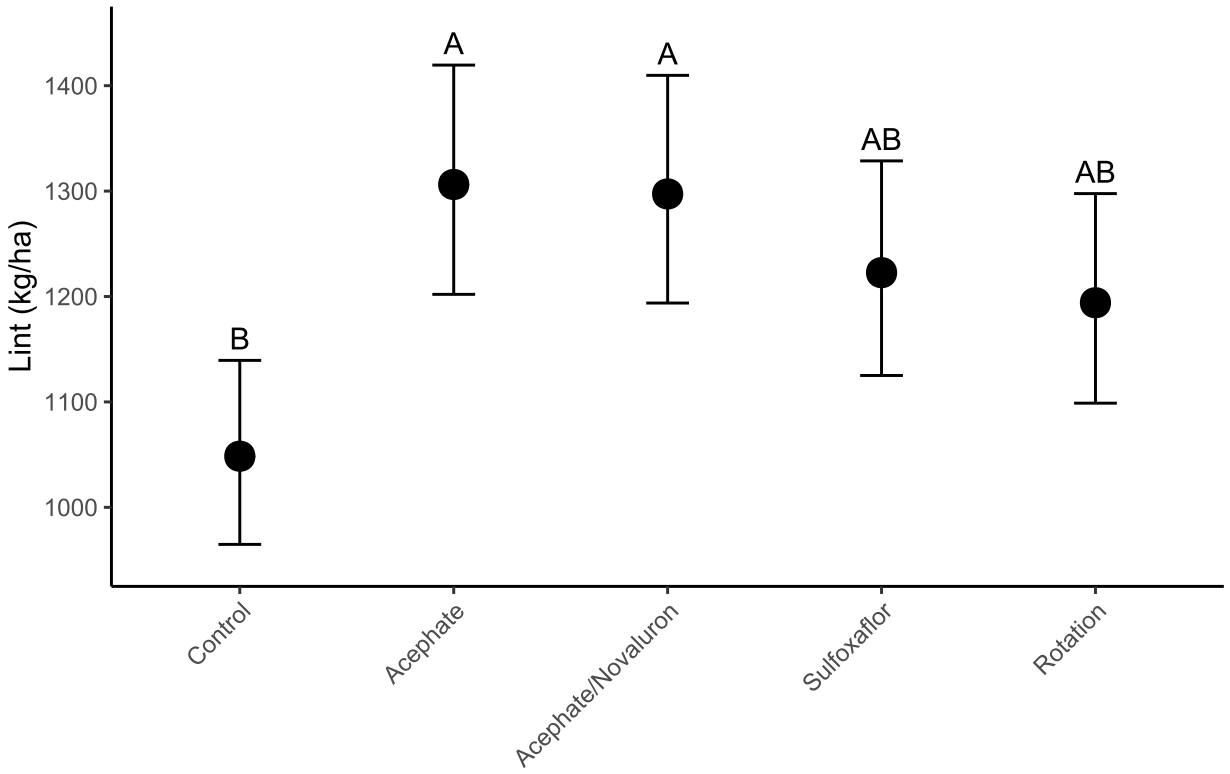

Figure 5.3. Estimated marginal mean (± 95% CI) of cotton lint. In Virginia, lint was weighed after harvest and ginning in Virginia. In North Carolina, combined seed lint weight was taken after harvest and assumed 40% lint weight. Lint weights were calculated from test plots in 2020 and 2021. Treatments and Standard error bars with the same letters are not significantly different ($P < 0.05$) using Tukey's HSD.

Chapter 6: Overall Conclusion

My research was driven by the recent prominence of *L. lineolaris* to the forefront of the pest complex in mid-Atlantic cotton during the last decade. Specific outputs of this dissertation included: 1) an assessment of cover crops and weed hosts as overwintering sites of *L. lineolaris*, 2) determined levels of carbohydrates, lipids, and proteins between reproductive and diapausing *L. lineolaris*, 3) identifying flight differences of *L. lineolaris* coming from different weed populations, and causes the dispersal from weed hosts to cultivated cotton, and 4) recommending insecticide rotations in cotton to limit the impact on natural enemy densities.

Research has explored the impact of weed hosts on overwintering *L. lineolaris* in the mid-south, while studies have not been performed to discover the influence of cover crops as an overwintering site for *L. lineolaris*. Understanding the implications of cover crops as winter hosts allows for the educated evaluation of cultural control methods such as weed removal and cover crop choice. Preferred weeds as overwintering hosts for *L. lineolaris* included hairy vetch and deadnettle species, suggesting the potential benefits of controlling these weeds. The implementation of cover crops can reduce weeds in cotton fields, but the choice of cover crop can be impactful on *L. lineolaris* as well. Grains could be considered, as populations of *L. lineolaris* had limited survivorship on wheat and rye, while legume cover crops like clover and vetch offer greater overwintering success.

Bioassay modification allowed for the assessment of metabolic changes within *L. lineolaris*, aiding in understanding the insect's biology, while defining methods to determine the

insect's diapause status. Assays were successfully modified to determine the full body quantity of carbohydrates, lipids and proteins within *L. lineolaris*. Diapausing insects had elevated carbohydrate and lipid levels, but lower protein levels. The bioassays also allow for the identification of diapause status in *L. lineolaris*, which will allow this nutrient quantification method to easily perform more diapause studies in the future.

The dispersal from weed hosts to mid-Atlantic cotton is an important event that is not well understood. While there were limited differences in flight potential between *L. lineolaris* from differing weed hosts, the starved control had the highest number of attempting and sustaining flight. Populations from weed hosts had higher pre-flight body weight and internal lipid levels that may have made them more fit to survive the flight mill or give them a higher flight potential. Interestingly, protein was higher in starved individuals indicating an upregulation in protein transcription. When exposed to cotton, populations from weeds were more likely to maintain high activity, while starved populations were more likely to settle on plants, implicating weed senesce as the factor driving *L. lineolaris* to cotton.

Insecticide trials indicated that tested active ingredients perform effectively in controlling *L. lineolaris* and other key pests in cotton, but certain insecticides are more prone to cause additional impacts on secondary pests and natural enemies. The combination of acephate with novaluron caused secondary pest spikes above the other treatments including acephate alone. Repeated acephate applications caused issues with secondary pest levels, while depleting natural enemy populations; however, populations were stable when acephate applications were rotated with selective insecticides. IPM practices will make it unlikely for multiple acephate applications

to occur within the same field, but insecticide rotation along in tandem with chemistries that support natural enemy populations should continue to be stressed to mid-Atlantic cotton growers.

As *L. lineolaris* continues to be a major economic pest to mid-Atlantic cotton, this research provided information on current control methods, as well as providing input on the insect's physiology to offer additional pest management strategies. Manipulation the insect's natural physiology may allow for the establishment of year-round reduction of *L. lineolaris* populations and decreased damage to mid-late summer cotton.