Insecticide resistance monitoring and sublethal effects of an insect growth regulator on tarnished plant bug (Hemiptera: Miridae)

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Insecticide resistance monitoring and sublethal effects of an insect growth regulator on tarnished plant bug (Hemiptera: Miridae)

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A Thesis
Submitted to the Faculty of Mississippi State University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Agricultural Life Sciences in the Department of Biochemistry, Molecular Biology, Entomology and Plant Pathology

Mississippi State, Mississippi

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Efforts to implement integrated pest management (IPM) strategies and improving our understanding of existing strategies are keys to achieving adequate control of tarnished plant bug, *Lygus lineolaris*. Insecticide applications are a part of IPM and monitoring their efficacy is critical for producers to remain profitable. Resistance to imidacloprid, thiamethoxam, and sulfoxaflor was documented, although there continues to remain variability among populations within the region. The insect growth regulator, novaluron, although only lethal to nymphs, plays an important role in management of *Lygus*. Sublethal impacts to adult plant bugs may be an important factor in reducing populations within the growing season. Future research to confirm the validity of using a laboratory colony as a baseline for insecticide susceptibility is needed to account for increased vigor of insects reared on artificial diet.
DEDICATION

Better is the end of a thing than its beginning, and the patient in spirit is better than the proud in spirit. Ecclesiastes 7:8
ACKNOWLEDGEMENTS

Mississippi State University provides a great opportunity for employees to better themselves by obtaining a higher formal education at little to no cost and I appreciate that opportunity. Working as a full-time employee and a part-time graduate student, it took a while for me to finish my degree, but we finally did it. I do mean “we” because I did not make it on my own.

A special thank you to my committee for your patience and endurance. As my advisor, Dr. Fred Musser, thank you for taking a chance on me and for keeping me and my research statistics squared away. Natraj Krishnan, you are a patient and kind individual, and the most stress-free person I know. Thank you for teaching me about ovarian dissections and mentoring so many others. Jeff Gore, you are like the small quiet voice, not talking a lot but when you speak, everyone listens. Your knowledge and expertise have helped so many. Ryan Jackson, I admire your willingness to stand for what is right even when it’s not popular and helping me find a research project of great importance to Mississippi’s agriculture community. Did I mention that I love your attitude?
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CHAPTER I
INTRODUCTION

Introduction

Since the late 1800s, *Lygus* spp. have been known as insect pests across the world in a variety of crops causing injury in wheat, corn, potatoes, nursery stock and strawberries (Cook 1876a, Forbes 1884a) as cited in New York State College of Agriculture report 1914. In addition to these crops, injury was found in mountain ash and fruit trees (Wier 1872, Ravn and Rasmussen 1996) and Hitchings (1908) reported injury specifically to apple buds and fruit. Some have even referred to *Lygus* as “a bad fruit bug” (Slinger 1895). Numerous vegetables, flowers, some conifers, and agronomic crops such as corn, cotton, and soybean are also host of this pest. Regardless of crop, with some variation depending on the host, host injury occurs, and the damage is seen through discoloration, growth reduction or distortion, and fruit abscission which affects overall crop production.

The tarnished plant bug (TPB), *Lygus lineolaris* (Palisot de Beauvois) (Hemiptera: Miridae), is an economically important pest of cotton, *Gossypium hirsutum* (Linnaeus) (Malvales: Malvaceae). Although TPB has always been a pest of cotton, with the success of boll weevil, *Anthonomus grandis grandis* (Boheman) (Coleoptera: Curculionidae) eradication and adoption of Bt, *Bacillus thuringiensis*, technologies, an overall decline of foliar insecticide applications that were consequently providing control of TPB, has allowed it to become the primary pest in cotton. Tarnished plant bug has been costly to midsouth cotton growers since that time (Cook
Several insect pest management practices, including rotation of different classes of insecticides are currently utilized to manage this pest and produce a profitable crop for growers. Over time, some insecticides have become less effective, requiring more applications and/or mixtures of active ingredients for adequate control. As expected, after using the same chemicals, alone or in combination with other chemicals for extended periods of time, an increased number of insecticide applications each year, and higher application rates, TPB has developed resistance to numerous insecticides. For those products that are still efficacious, establishing baselines and monitoring populations for susceptibility and resistance is important to detect changes before experiencing field control failures.

Insect growth regulators (IGR) include insecticides with several modes of action that are commonly used in cotton in the midsouth; however, all of them only control the immature stages of insects. Because of this IGs are commonly mixed with adulticides to provide control of both nymphs and adults. Novaluron is the most commonly used IGR for TPB in cotton in the midsouthern region. In addition to killing immature insect stages, insect growth regulators have been shown to reduce egg hatch rate in some Lepidoptera (codling moth, *Cydia pomonella* (Linnaeus)), Coleoptera Colorado potato beetle, *Leptinotarsa decemlineata* (Say)), and Diptera (common house mosquito, *Culex pipiens* (Linnaeus)) when adults were exposed (Cutler et al. 2005a, Alyokhin et al. 2009, Kim et al. 2011, Djeghader et al. 2014). Insecticides are currently a major part of cotton pest management, so monitoring the effectiveness of those insecticides and learning their impact on pests and the production of cotton is important. Establishing a baseline followed by resistance monitoring and a complete understanding of the impacts of these chemicals will be key to sustainable, successful management.
Cotton

Basics of Cotton

Although we do not know precisely how long cotton has been used by humans, it has been found in caves in Mexico that scientists believe to be 7000 years old. There is also evidence of cotton being spun and woven in Pakistan as early as 3000 BC. The Europeans had cotton from as early as 800 AD and by the 1500’s it was generally found throughout the world (National Cotton Council). The invention of the cotton gin made it possible to supply large amounts of cotton fiber to the textile industry. In the early 1800’s, the annual value of the United States cotton crop rose from $150,000 to over $8 million (National Cotton Council). Today, the U.S. is the 3rd largest cotton producing country and the largest exporter in the world. There are several species of cultivated cotton, each with different characteristics and grown in different regions. *Gossypium barbadense* (Pima cotton) and *Gossypium hirsutum* are grown the U.S. *G. hirsutum* (Upland cotton) is the only type of cotton grown in the midsouthern U.S. and accounts for about 90% of cotton production worldwide (YARA World Cotton Production).

Growth and Development

Cotton is grown in warmer climates because it requires many heat units to reach maturity. Cotton is an indeterminant plant, meaning that vegetative growth continues after reproductive growth has begun (Silvertooth et al. 1999). Under good growing conditions in the midsouth, cotton will continue to produce flowers for up to eight weeks, making it susceptible to pests of reproductive structures for a long time. Other crops generally do not have such an extended flowering period.

Growth and development of cotton is measured in degree days (DD) or heat units to determine how long a particular stage lasts as this can vary in calendar days depending on the
temperature. Degree days are calculated by adding the daily high to the daily low temperature, dividing by 2 and subtracting the threshold for cotton growth (60°F) (Landivar and Benedict 1996). In the midsouth, mature cotton requires 2200-2600 heat units (measured in °F) after planting, which is typically 130-160 days. The main growth stages are germination and emergence, seedling, leaf and canopy development, flower and boll development, and maturity (Jenkins et al. 1990). Because of cotton’s indeterminate growth habit and long growing season it is vulnerable to many pests for an extended period of time and effective management strategies are needed to protect yield and profit (Oosterhuis 1990).

**Insect Pests of Cotton**

There are numerous insect pests that attack cotton, but only about 1/4th of them cause economic loss annually in the midsouth region (Leigh et al. 1996). Some of the more common pests of cotton in the midsouth region are thrips, *Franklinella fusca* Hinds and *F. occidentalis* Perganda (Thysanoptera: Thripidae), twospotted spider mite, *Tetranychus urticae* Koch (Arachnida: Acari), cotton aphid *Aphis gossypii* (Linnaeus) (Hemiptera: Aphididae), tarnished plant bug, and the heliothine complex of *Helicoverpa zea* (Boddie) and *Chloridea virescens* (F.) (Cook 2019). Thrips cause delays in maturity by delaying plant growth and development in the seedling stage which reduces yield in some circumstances (Stewart et al. 2013). Twospotted spider mite is an occasional pest of cotton that cause damage when in high numbers by feeding on the undersides of leaves, often leading to chlorosis and even premature defoliation which can reduce yields. *Helicoverpa zea* and *C. virescens* feed on nearly all plant parts but primarily are pests of developing fruit. Fruit fed on by heliothines often abscise and fall off the plant leading to direct yield loss. While heliothine are the primary pests on traditional (conventional) cotton, most cotton grown in Mississippi currently is transgenic, including proteins derived from
Bacillus thuringiensis Kurstaki (Berliues) which provides substantial control of the heliothine complex. Tarnished plant bug is consistently the most economically important pest of transgenic cotton in the midsouth. They feed on terminals, squares, flowers, and bolls. On cotton, they prefer to feed on the squares but will feed on almost any part of the cotton plant (Leigh et al. 1996). Tarnished plant bugs can cause substantial yield loss under high populations. Although there are several plant bug species that occur in cotton globally, Lygus lineolaris is the main species encountered in the midsouth (Young 1986).

Lygus lineolaris

Biology and Ecology

Lygus lineolaris is a true bug in the family Miridae (Hemiptera) (Triplehorn and Johnson 2005), and is found in all Canadian provinces, the United States, and many of the states in Mexico (Kelton 1980, Young 1986). In the United States, more than half of cultivated plant species are hosts plants for TPB (Capinera 2001). Tarnished plant bugs overwinter as adults and can be found in leaf litter and tree bark. Henbit, Lamium amplexicaule (Linnaeus) and crimson clover, Trifolium incarnatum (Linnaeus) are also important overwintering plants (Snodgrass et al. 1984). Additionally, flea bane, Erigeron annus L., has been reported as a well-known spring host (Cleveland 1982). Spring populations build in wild host plants in roadside ditches and field edges for one to two generation or two before wild hosts senesce, and then they move to other flowering hosts (Fleischer and Gaylor 1987). Multiple generations occur throughout each growing season in the southern U.S.

Tarnished plant bugs complete a gradual metamorphosis that consists of three life stages: eggs, nymphs, and adults. Eggs are laid singly and are ~ 1mm in length and 0.25mm wide and are slightly curved with a flat top opening and hatch within 7-10 days (Capinera 2001).
Tarnished plant bug eggs are generally inserted into host plant tissue by the adult female. The wingless nymphs are ~1-4mm in length and light yellowish to green in color. There are five nymphaal instars. As they progress through the various instars, bands and spots develop on the legs, thorax, and abdomen with the last nymphaal instar developing wing pads. It takes roughly 30 days to complete a life cycle. Adults are 5-6mm in length, dark brown in color with comparatively long antennae and legs (Fleischer and Gaylor 1987). Ambient temperature will cause the length of the life cycle to fluctuate (Ugine 2012).

Tarnished plant bugs have piercing and sucking mouth parts. They are highly polyphagous, having over 700 documented plant hosts (Parys and Snodgrass 2014). Young (1986) said *Lygus* have the widest host range of any insect. Layton (1995) stated that movement of this pest appeared to correlate with the number of alternate hosts available and the flower buds on those hosts.

**Feeding in Cotton**

As TPB feed, salivary enzymes are injected into the plant, which causes the plant tissue to break down. These enzymes are responsible for causing more damage than the amount of tissue that is consumed while feeding (Layton 1995). During the past eleven years (2009-2019) growers in the Mississippi Delta region averaged 3.5-7 applications annually to control this pest. Average cost of insecticide applications ranged from $85.58 – $239.64/hectare ($34.65 – 97.02/acre) which was an average of 57% of all insecticides applied in cotton during that time with highest percent 2009 (72%) – lowest percent 2019 (38%) (Cook 2020).

Tarnished plant bugs can begin to damage cotton shortly after seedling emergence and may continue through early lint development of the last harvestable bolls. Scales and Furr (1968) discuss “crazy cotton” damage. This occurs when plant bugs feed on tissue in the terminal
of pre-squaring cotton, causing a loss of apical dominance and resulting in multiple secondary terminals.

Most economic damage is thought to occur from first square through the early bloom stages (Black 1973). Feeding may cause enlarged nodes, aborted terminals, unnecessary and excessive branching near the main stem, and fruit delay, which all can lead to yield reductions (Hanny et al. 1977). Tarnished plant bugs prefer to feed on squares less than 3.18 mm (1/8 in.) in diameter in cotton rather than large squares and bolls (Tugwell et al. 1976), which causes abscission of the small squares (Layton 1995). Having excessive early square loss from TPB feeding may also cause a delay in crop growth and fruiting, leading to delayed maturity.

Layton (1995) stated *L. lineolaris* damage remains localized to the feeding area when large squares or bolls are fed on. Feeding on larger squares seldom causes the square to abort, but the square will bloom, and damage is normally observed as damaged anthers and referred to as “dirty blooms.” In 1976, Pack and Tugwell reported a correlation between discolored anthers and boll damage. When over 30% of anthers are damaged, the level of malformed bolls and percent of boll shed increases. Tarnished plant bugs also feed on small bolls which leads to sunken lesions that cause the outside of the boll to turn black and necrotic. On larger, more developed bolls, individual seeds are damaged. This causes lint to be discolored and overall boll weight decreases (Pack and Tugwell 1976).

**Thresholds and Sampling**

Sampling with a sweep net is very effective for adult population monitoring and the drop cloth is more effective when monitoring nymphs (Musser et al. 2009). Also, visual sampling is sometimes used, particularly if the cotton has excess growth making sweep nets and drop cloth methods difficult to conduct (Musser et al. 2009). Depending on the developmental stage of
cotton, thresholds vary, increasing with more mature cotton (Craig 1998, Catchot et al. 2020). The current threshold for Mississippi between cotton emergence and first square, using a visual sampling, is five TPB per 100 terminals. Using a sweep net during the first two weeks of squaring, the threshold is eight per 100 sweeps, and using a black drop cloth it’s one plant bug per 1.5 m of row. During the third week of squaring the thresholds double except for the drop cloth where the threshold goes to three TPB per 1.5 m of row (Musser et al. 2009). No threshold exists for percent dirty blooms; however, the presence of dirty blooms is an indication that TPB were previously present and they were feeding on larger squares (Catchot et al. 2020).

**Management Practices**

Integrated pest management practices are critical to provide acceptable control of tarnished plant bug. Utilizing all available strategies into a unified management approach backed by weekly or biweekly scouting has led to some success in controlling this pest in the midsouth region (A. Catchot, Per. Comm). Timeliness of insecticide applications are critical. Musser et.al (2009) reports an individual TPB caused the loss of 0.6-2.1 squares per day. Planting early and planting early maturing varieties has shown a positive impact on yield by avoiding late season populations and it often reduces the total number of insecticide applications needed (Adams 2013). Combining multiple pest management practices together using hairy leaf upland cotton varieties (Meredith and Schuster 1979, Bailey et al. 1980, Wilson and George 1986, Woods et al. 2017), a reduced nitrogen rate (Samples 2014) and a novaluron application during the third week of squaring (Gore et al. 2010) may be beneficial. Graham (2016) tested all of these techniques together and was able to protect yield and reduce the total number of spray applications for TPB in the MS Delta region. Although implementing all known strategies into a
unified IPM program to control TPB in cotton proved beneficial, insecticides are still a crucial component of the program (Graham 2016).

**Insecticides**

Commonly used foliar insecticides to control TPB include: Sulfoximines such as sulfoxaflor (Transform WG, Dow AgroSciences, Indianapolis, IN), organophosphates like acephate (Orthene 90S, Amvac Chemical Company, Walnut Creek, CA) and dicrotophos (Bidrin 8E, Amvac Chemical Company, Walnut Creek, CA), and chloro-nicotinoyl’s such as thiamethoxam (Centric 40WG, Syngenta Crop Protection, Greensboro, NC) and imidacloprid (Admire Pro 4.6SC, Bayer CropScience, Research Triangle Park, NC). Novaluron (Diamond 0.83 EC, Chemtura USA Corporation, Middlebury, CT), an insect growth regulator, is also commonly used but is only lethal on plant bug nymphs and must be combined with an adulticide to provide control of all stages.

Many of these compounds are used either alone or mixed with other insecticides. These classes of chemistry are often rotated because of their different modes of action. Sulfoximines and chloro-nicotinoyl’s are nicotinic acetylcholine receptor agonists and behave similarly, causing hyper-excitation leading to paralysis. Organophosphates are acetylcholinesterase inhibitors and terminate the action of the neurotransmitter acetylcholine at nerve synapses. These fast-acting chemicals target the nerves and muscles (IRAC 2020). Although pyrethroids are not used as extensively in the MS Delta region because of resistance, they are still used in other areas and kill insects by keeping the sodium channels open which causes a hyperexcitation and in some cases nerve block. These sodium channels are involved in the propagation of action potentials along the nerve axons (IRAC 2020).
There are two insect growth regulators commonly used in MS that are classified as chitin synthesis inhibitors: novaluron and diflubenzuron. Novaluron is commonly used in cotton to control *Lygus* and fall armyworm, *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae). Diflubenzuron is more commonly used in soybean, *Glycine max*, to control defoliating caterpillars such as velvetbean caterpillar, *Anticarsia gemmatalis* (Hübner) (Lepidoptera: Erebidae) and green cloverworm, *Hypena scabra* (Fabricius) (Lepidoptera: Erebidae). A third insect growth regulator used in MS is methoxyfenozide and is used most often in soybean to control soybean looper *Chrysodeixis includens* (Walker) (Lepidoptera: Noctuidae). Methoxyfenozide mimics the molting hormone, ecdysone and accelerates the molting process. Although not an insect pest, two-spotted spider mites are considered a regular pest of cotton in the midsouth region and are also often targeted with the insect growth regulator, etoxazole. Etoxazole inhibits the enzyme that catalyzes the polymerization of chitin. Typically, insect growth regulators are slow acting and disrupt or prevent metamorphosis (IRAC 2020). Because of their unique qualities interfering with metamorphosis or chitin synthesis, they only control the immature stages of the insects and have to be tank mixed with other insecticides to control the adult stages.

**Thesis Rationale**

**Thesis rationale**

In 1992, the first pyrethroid resistance in TPB was documented in the MS Delta (Snodgrass 1994). Organophosphate resistance has also been documented (Snodgrass 1996, Snodgrass et al. 2009). Late instar nymphs are more tolerant to numerous insecticides than adults (Hollingsworth et al. 1997, Allen et al. 2012). With reduced insecticide applications
targeting other pests and reduced efficacy of existing insecticides, insecticide rates and application targeting TPB has increased over time (Cook 2020).

Because multiple insecticide applications are commonly made annually in cotton grown in midsouth and there is documented resistance to several classes of insecticide there is a threat of further resistance development, so resistance monitoring remains important. Expanding our knowledge on sublethal impacts of insect growth regulators and how they affect tarnished plant bug population dynamics may also improve management strategies and reduce insecticide applications. To document current resistance levels to common insecticides and improve our understanding of insect growth regulators, the following objectives were addressed:

**Objective 1:** To quantify levels of resistance in TPB populations with commonly used insecticides in the midsouth region.

**Objective 2:** To determine the impact of adult exposure to novaluron on TPB population dynamics.
References


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CHAPTER II
RESISTANCE MONITORING OF INSECTICIDES TO TARNISHED PLANT BUG POPULATIONS IN THE MIDSOUTHERN USA

Abstract

As tarnished plant bug populations were tested to quantify levels of resistance with commonly used insecticides, data supports an overall finding 23-36% of populations are resistant. Resistance monitoring in the midsouth from 2017-2019 showed no differences in imidacloprid and thiamethoxam LC$_{50}$ values by year, however for sulfoxaflor, the mean LC$_{50}$ was higher in 2019 than in 2017. Comparisons of the percentage of populations with resistance ratios greater than 10 indicates that for the Delta and Hills regions each year, between 23% and 36% of the populations were considered resistant to all three insecticides. As reflected in the mean LC$_{50}$ data, the percentage of resistant populations were similar each year for imidacloprid and thiamethoxam, but for sulfoxaflor the percentage of populations considered resistant increased each year, reaching 75% during 2019. For each insecticide, variability between the most and least susceptible populations was greatest in the Hills region and during 2018. Thiamethoxam had the most variability followed by imidacloprid and sulfoxaflor.

Introduction

_Lygus lineolaris_ (Palisot de Beauvois) has been a major pest of cotton in the midsouth United States for years (Catchot et al. 2009). With the damage and yield losses caused by this pest, management of this insect is essential in nearly all fields every year. While planting early,
choosing short-season varieties, and keeping the field edges clean from wild hosts are helpful (Adams et al. 2013), management of this pest often requires multiple applications of insecticides from various chemical classes to optimize profit (Cook 2019). Organophosphate, carbamate, neonicotinoid, and insect growth regulator insecticides are used to control multiple cotton pests like thrips, spider mites, aphids, the heliothine complex, and plant bugs (Cook 2019). For control of plant bugs, neonicotinoid (imidacloprid and thiamethoxam) and sulfoximine (sulfoxaflor) insecticides are generally applied first followed by organophosphate insecticides later in the season (Catchot et al. 2019). Currently tank mixes of two or more chemical classes at the highest labeled rates are recommended to provide control (Catchot et al. 2019). Regardless of chemical class, after years of multiple insecticide applications and high application rates, the presence of insecticide-resistant populations of *L. lineolaris* is not surprising. Establishing a baseline of susceptibility is a key factor for monitoring for resistance.

The first pyrethroids were used to control flying pests of domestic animals and household insects, ectoparasites, as well as pharmaceutical products for lice and scabies (Henault-Ethier 2015). Although having high insecticidal activity they were easily oxidized under ultraviolet light. Later more photostable pyrethroids with higher insect toxicities were developed in the 1960-1970’s. Being used heavily in many different areas, a concern with these second generation pyrethroids was the development of resistance (Melnivok 1971). Organophosphates were used mainly for residential purposes during the late 1970’s (Feo et al. 2010). With the banning of many of the uses of organophosphates due to concerns of human toxicity by the Environmental Protection Agency (EPA) (Metcalfe et al. 2002), eventually, many of their uses were replaced with pyrethroids (Oros and Werner 2005). Pyrethroids were readily adopted because of their high efficacy and broad spectrum of control. Snodgrass (1994) reported that
after 14 years of pyrethroid use on cotton, *L. lineolaris* resistance was present at a Mississippi location and then across the midsouth (Snodgrass 1996a). Since that time, multiple studies have reported populations resistant to pyrethroids, as well as some carbamates and organophosphates (Snodgrass 1994, Snodgrass 1996a, Snodgrass and Scott 2000, Pankey et al. 1996, Hollingsworth et al. 1997, Snodgrass et al. 2009, Parys et al. 2017). To help combat resistance and provide another mode of action, neonicotinoids became widely used in cotton in the late 1990’s and a couple of decades later there were reports of field populations of *Lygus* with increased LC50s of imidacloprid and thiamethoxam. With the introduction of sulfoxaflor in 2012, shortly thereafter an initial baseline was established for sulfoximines (sulfoxaflor) (Parys et al. 2017).

In this paper we provide new resistance monitoring results for *L. lineolaris* with sixty-five populations collected during 2017-2019 from two well defined geographical regions within the midsouthern U. S., the Delta and Hills (Figure 1). The Delta has a larger proportion of cultivated area with larger individual fields that are in close proximity (NASS 2017). Agricultural fields in the Hills generally are smaller, not in close proximity to each other, and overall, make up a smaller percentage of the landscape (NASS 2017). Both regions grow cotton and have *L. lineolaris*, but *L. lineolaris* pressure, and therefore insecticide applications, tends to be greater in the Delta region (Fleming et al. 2015, Cook 2019). Populations from both regions were collected during 2017-2019 and assayed with thiamethoxam, imidacloprid and sulfoxaflor and compared to a laboratory colony. These data combined with already published data (Parys et al. 2017) will be useful in documenting changes in susceptibility to these products over time.
Materials and Methods

Laboratory colony

A colony of *L. lineolaris* established in 2005 at Mississippi State University was used for several of the experiments described below. This colony was collected from uncultivated hosts in MS and had periodic infusions of wild *L. lineolaris* numerous times since establishment. The colony was reared in 40 cm x 25 cm x 13 cm plastic containers as described by Musser et al. (2012) and maintained at 27°C, 70% relative humidity with a 16:8 L: D cycle. In 2017, the light cycle was changed to 14:10 L:D. The colony was fed a semi-solid oligidic diet (Cohen 2000) that also included 33.6 ppm fumagillin (Musser et al 2012). Diet was presented in Parafilm® (Pechiney Plastic Packaging, Menasha, WI) packets and changed three times per week. Egg packets were made with a 4% carrageenan solution in a Parafilm® packet, placed on the top of the rearing containers, and changed three times per week.

Field collections.

Adult tarnished plant bug populations were collected from uncultivated flowering plants (e.g., daisy fleabane) throughout Mississippi, Tennessee, Arkansas, and Louisiana cotton growing regions with sweep nets during May-August 2017 - 2019. Insects were aspirated into containers and fed fresh green beans or host plants until the assays could be conducted. Insects were placed in a vehicle and driven the same day collected to Mississippi State University. Assays were conducted within 48 h of collection.

Bioassays.

All assays were conducted using 20 ml glass scintillation vials. Prior to use in assays, vials were submerged in a bleach water solution of 240 ml 7.5% ai sodium hypochlorite /18.9 liters water (1
cup/5 gallons) for 2 days, individually triple rinsed with tap water, placed upside down in a vial rack and baked at 149°C (300°F) for 3 hours. After vials cooled to room temperature, they were removed from the oven, rinsed with acetone and placed and vials placed in a chemical fume hood until dry.

Assay methodology was based on insecticide mode of action. Susceptibility to sulfoxaflor, primarily a contact insecticide (Parys et al. 2017), was assayed using a coated vial. 250 µl of a sulfoxaflor-acetone solution prepared in five concentrations ranging from 0.1 to 31.6 µg sulfoxaflor/vial or a control of pure acetone was dispensed into each vial. The vial was then placed immediately on an unheated hotdog roller in a chemical fume hood and rolled until dry. Vials were treated within 24 h of beginning the assay. A surface-sterilized piece of fresh green bean was added to each vial as a food source. Two *L. lineolaris* adults were placed in each vial and the vial was capped with a cotton ball. Vials were kept at room temperature and mortality was assessed after 24h (Parys et al. 2017).

Imidacloprid and thiamethoxam are most active through ingestion, so these insecticides were tested using floral foam (Snodgrass et al 2009). Using a cork borer, a 12 mm x 102 mm plug was removed from a block of “wet style” Oasis floral foam and cut into round disks measuring 12 mm x 12 mm. A floral foam disk was placed inside each vial and 0.5 ml of a 10% honey water solution containing one of five insecticide concentrations ranging from 0.1 to 31.6 µg/ml of solution or a control of 10% honey water solution was pipetted carefully into the floral foam so that no droplets were outside the floral foam. A single *L. lineolaris* adult was placed in each vial and the vial was capped with a cotton ball. Vials were kept at room temperature and mortality was assessed after 24 h (Parys et al. 2017). Approximately 30 insects were tested at each concentration for all assays.
Data Analysis.

Data were analyzed using probit analysis (PROC PROBIT, SAS 9.4, SAS Institute; Cary, NC) to estimate an LC$_{50}$ for each assay. Those assays with a good fit to the probit model (chi-square goodness of fit $P>0.05$) and a significant response to insecticide concentration ($P<0.05$) were used for further analysis. Using the LC$_{50}$ estimate for each of these assays, analysis of variance (PROC GLIMMIX, SAS 9.4) was conducted to evaluate whether region or year were significant factors. The identity link function and the Gaussian distribution were used, and degrees of freedom were calculated using the Kenward-Roger method. Fisher’s Protected LSD test with $\alpha=0.05$ was used to separate means. Means and standard errors were determined using PROC MEANS (SAS 9.4).

Previous research groups routinely collected field populations from Crossett, AR as the susceptible colony although Parys et al. (2017) was concerned about its sustainability (Snodgrass 1996a, Snodgrass and Scott 2000, Snodgrass et al. 2008a, Snodgrass et al. 2009, Parys et al. 2017, Parys et al. 2018). The Crossett location has pine and timber production and no row crops in the area. We were unsuccessful in making a collection from Crossett, AR for this study. Furthermore, using a new field collection for a baseline is not ideal because the genetics may change over time and results tend to be more variable than results from a laboratory colony (Parys et al. 2017). While the laboratory colony used in this manuscript provided consistent data, it does not appear that it represents a baseline for *L. lineolaris* insecticide susceptibility because the LC$_{50}$s for the laboratory colony was generally higher than for the field populations. Since the field populations were tested within 48 h of collection, the health and vigor of the field populations were not comparable to the laboratory colony. This could be linked to consistent rearing conditions and the nutritional value of artificial diet compared to wild hosts. These
factors have been shown to affect susceptibility in Lepidoptera and other insect pests (Gordon 1961, Wood et al. 1981, Jensen et al. 2016). In addition to trying to collect a susceptible field population from Crossett, AR, we also tried to rear our laboratory colony on broccoli to reduce the size and fat content and more closely mimic the nutritional condition of field-collected populations. While we could rear some to adulthood, we could never produce enough adults to conduct bioassays.

Because the conventionally reared laboratory colony produced consistent results in the assays, we are confident that the assay methodologies produce repeatable data and that the differences observed between populations were largely a result of differences in the susceptibility of the populations. Therefore, we created an unconventional baseline by taking the mean of the 5 lowest field population assays for each insecticide as the baseline of a susceptible population. Comparing the lowest five assays to the laboratory colony assays showed the laboratory colony to be 12, 19, and 26 times more resistant for thiamethoxam, imidacloprid, and sulfoxaflor, respectively (Table 1). One would expect this ratio to be consistent if the reduced susceptibility of the laboratory colony is a function of rearing conditions, but variable if the genetics of resistance were important factors. Because the ratio of the laboratory colony to lowest field populations was fairly consistent over all three insecticides, we believe this method for establishing a baseline for insecticide susceptibility was reasonable and provides a useful method of evaluating the development of resistance in field populations. Resistance ratios (RR) were calculated by dividing the LC50 of each population by the mean LC50 of the 5 lowest field populations for each compound tested.

Variability in LC50 values of individual populations within a region or year was determined by dividing the highest LC50 by the lowest LC50. High variability may indicate
variable resistance levels within the parameter being measured, while low variability suggests no resistance or fixed resistance in all populations.

Results
The proportion of usable assays to total assays was 53% during 2017, 68% during 2018, and 79% during 2019. Usable data over all three insecticides and three years totaled 67 assays on field populations and 9 assays on the laboratory population (Appendix 1). Multiple collections within the same counties were made in both regions and years of study. LC50 estimates for field populations ranged from 0.57 to 33.82 ppm for sulfoxaflor, 0.03 to 9.27 ppm for imidacloprid, and 0.004 to 13.14 ppm for thiamethoxam. LC50 estimates for the laboratory population ranged from 9.42 to 35.33 ppm for sulfoxaflor, 2.23 to 3.41 ppm for imidacloprid, and 0.57 to 0.77 ppm for thiamethoxam.

Mean LC50 values did not differ for any compound tested with respect to region or laboratory colony (Table 1). For imidacloprid and thiamethoxam there were no differences for LC50 by year, however for sulfoxaflor, the mean LC50 was higher in 2019 than in 2017, with 2018 being intermediate. Comparisons of the percentage of populations with resistance ratios greater than 10 shows that for the Delta and Hills regions each year, between 23% and 36% of the populations were considered resistant to all three insecticides. Yearly fluctuations were similar, ranging from 11-44% except for sulfoxaflor during 2019 when 75% of the populations were considered resistant.

Using the lowest field colony LC50 estimates as the baseline for susceptible populations, all assays on the laboratory colony with all three insecticides resulted in resistance ratios >10 (Table 1), even though the laboratory population was not exposed to any insecticide in more than
10 years, which is longer than sulfoxaflor has been on the market (Environmental Protection Agency 2019).

For each insecticide, variability was greatest in the Hills region and during 2018 (Table 1). Thiamethoxam had the most variability (980) followed by imidacloprid (309) and sulfoxaflor (54). As evidence that the assay methodology was consistent, low variability was observed for the laboratory colony, ranging from 1.4 - 3.8 for all insecticides.

**Discussion**

The Delta region is farmed more intensively and annually tends to have higher insect pressure. More insecticide applications are made in the Delta region than in the Hills region (Fleming et al. 2015). Snodgrass and colleagues, primarily testing populations from the Delta region, showed resistance of *L. lineolaris* to insecticides in multiple classes: pyrethroids (Snodgrass 1994, Snodgrass 1996b), carbamates and organophosphates (Snodgrass and Scott 2000, Snodgrass et al. 2008a), and neonicotinoids (Snodgrass et al. 2008b). Although resistance ratios commonly exceeded 10, LC$_{50}$ values were generally within ranges previously reported throughout the midsouth. Similar to our results, Parys et al. (2017) generally found high levels of variability in susceptibility of *L. lineolaris* populations across the midsouth region. They concluded that most populations were susceptible to neonicotinoids, but several populations exhibited high levels of resistance. For sulfoxaflor, our results were also similar. They reported min./max. LC$_{50}$ values of 0.26/ 45.82, whereas we reported 0.57/ 33.82. Dorman et al. (2020) also reported slightly elevated resistance ratios in North Carolina and Virginia with sulfoxaflor. However, thiamethoxam resistance ratios were not different from the laboratory colony. Contrary to expectations, resistance was equally frequent in the Delta and Hills regions for all insecticides. The emerging resistance to sulfoxaflor is concerning as it is a major insecticide being currently
used, and the frequency of resistance increased each year, with 75% of the populations being considered resistant during 2019.

While it is unknown if current levels of resistance result in reduced field efficacy, it is likely that selection for resistance has already led to the large differences in susceptibility observed among populations with imidacloprid and thiamethoxam, however, this has not been reported with sulfoxaflor. It is important to continue monitoring these insecticides over time, as well as develop new chemistries, because continued selection for resistance is likely to lead to widespread reduced efficacy from all these pesticides in the future. How rapidly this will occur depends on the intensity of selection, the amount of movement between selected and unselected populations, and any fitness costs associated with resistance.

One of the interesting findings in this study is that the laboratory colony appeared to be more resistant than many of the field populations. As previously mentioned, this is likely due to the greater fitness of the laboratory colony. Because individuals reared in the laboratory are readily accessible and variability was low between assays, further research should be directed toward development of a rearing compensation factor to include for calculating resistance ratios. Based on our research using the five lowest populations per compound as the susceptible baseline, it appears that the laboratory LC\textsubscript{50} should be reduced in the range of 12-26-fold to calculate a fresh field colony susceptible colony equivalent. Establishing this baseline with a laboratory colony reduces variability inherent in field populations. Furthermore, as insecticides continue to be used across the landscape and average LC\textsubscript{50}s continue to increase, finding sufficient susceptible field populations to use as baseline data may become difficult.
Table 2.1  Mean LC$_{50}$ estimates for imidacloprid, sulfoxaflor and thiamethoxam by region and year for *L. lineolaris* collections made from the Midsouthern US during 2017-2019. Laboratory Colony (2019) included.

<table>
<thead>
<tr>
<th>Variable</th>
<th>N</th>
<th>LC$_{50}$ (ppm)</th>
<th>Resistance Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (SEM)</td>
<td>Lowest</td>
<td>Highest</td>
</tr>
<tr>
<td>Imidacloprid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Region: Delta</td>
<td>7</td>
<td>1.22 (0.37) A</td>
<td>0.08</td>
</tr>
<tr>
<td>Region: Hills</td>
<td>13</td>
<td>1.56 (0.71) A</td>
<td>0.03</td>
</tr>
<tr>
<td>Region: Lab</td>
<td>3</td>
<td>2.99 (0.38) A</td>
<td>2.23</td>
</tr>
<tr>
<td>Year: 2017</td>
<td>7</td>
<td>2.64 (1.21) a</td>
<td>0.45</td>
</tr>
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<td>Year: 2018</td>
<td>9</td>
<td>0.79 (0.25) a</td>
<td>0.03</td>
</tr>
<tr>
<td>Year: 2019</td>
<td>7</td>
<td>1.73 (0.54) a</td>
<td>0.24</td>
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<tr>
<td>Sulfoxaflor</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Region: Delta</td>
<td>11</td>
<td>9.50 (2.79) A</td>
<td>0.57</td>
</tr>
<tr>
<td>Region: Hills</td>
<td>10</td>
<td>7.31 (3.28) A</td>
<td>0.63</td>
</tr>
<tr>
<td>Region: Lab</td>
<td>3</td>
<td>20.15 (7.80) A</td>
<td>9.42</td>
</tr>
<tr>
<td>Year: 2017</td>
<td>8</td>
<td>2.84 (1.31) b</td>
<td>0.57</td>
</tr>
<tr>
<td>Year: 2018</td>
<td>9</td>
<td>10.12 (3.61) ab</td>
<td>1.08</td>
</tr>
<tr>
<td>Year: 2019</td>
<td>7</td>
<td>17.75 (4.10) a</td>
<td>3.08</td>
</tr>
<tr>
<td>Thiamethoxam</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Region: Delta</td>
<td>9</td>
<td>2.38 (1.50) A</td>
<td>0.09</td>
</tr>
<tr>
<td>Region: Hills</td>
<td>15</td>
<td>0.70 (0.28) A</td>
<td>0.004</td>
</tr>
<tr>
<td>Region: Lab</td>
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<td>0.57</td>
</tr>
<tr>
<td>Year: 2017</td>
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<td>2.61 (1.47) a</td>
<td>0.07</td>
</tr>
<tr>
<td>Year: 2018</td>
<td>8</td>
<td>0.80 (0.47) a</td>
<td>0.004</td>
</tr>
<tr>
<td>Year: 2019</td>
<td>10</td>
<td>0.40 (0.08) a</td>
<td>0.05</td>
</tr>
</tbody>
</table>

N= number of good fit populations.
LC$_{50}$ values reported in parts per million of active ingredient. LC$_{50}$s followed by same letter within an insecticide and variable type are not significantly different (Fisher’s Protected LSD [a=0.05]).

Resistance Ratios calculated by dividing the average LC$_{50}$ for per colony by the mean average of the 5 lowest field colonies’ LC$_{50}$s per chemical. Average LC$_{50}$ of 5 lowest field colonies per chemical: Imidacloprid (0.19), Sulfoxaflor (0.85) Thiamethoxam (0.05)

Variability: Highest LC$_{50}$/lowest LC$_{50}$.
RR > 10 is the % of populations tested with an estimated resistance ratio of at least 10.

Statistics for Region. Imidacloprid (F=0.80; df= 2,20, P>0.46), Sulfoxaflor (F= 1.84; df= 2,21; P>0.18), Thiamethoxam (F=1.16; df= 2,24; P>0.33).

Statistics for Year. Imidacloprid (F=1.72; df=2,20; P>0.21). Sulfoxaflor (F=5.01; df=2,21; P>0.02). Thiamethoxam (F=1.83; df=2,24; P>0.18).
Figure 2.1  Midsouth TPB collection county sites by region for each state from 2017-2019. Multiple collections were made within each county site except in AR.
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https://www.entomology.msstate.edu/resources/2019loss.php


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CHAPTER III
SUBLETHAL IMPACTS OF NOVALURON ON TARNISHED PLANT BUG (HEMIPTERA: MIRIDAE) ADULTS


Abstract
Tarnished plant bug, Lygus lineolaris Palisot de Beauvois (Hemiptera: Miridae), has become a primary pest of cotton in the Midsouthern United States. Insect growth regulators such as novaluron are an important part of L. lineolaris management. While novaluron is lethal to nymphs, it does not kill adults, so it has been used when nymphs are the primary stage present. However, cotton yield protection was observed from an application of novaluron when adults were the predominant stage present. To explain this, a series of studies were conducted to examine sublethal impacts of novaluron to L. lineolaris adults. Novaluron ingestion by adults reduced hatch rate and sometimes reduced oviposition rate. Ingestion by either males or females reduced hatch rates, but the reduction was greater from female exposure. Contact exposure of adults with novaluron residues within 1 d of application reduced hatch rate by about 50%, but the impact on oviposition was inconsistent. A field study showed reduced hatch rate from contact exposure to mixed-age natural populations, but the overall net reproductive rate was not reduced.
Surface exposure of eggs to novaluron did not reduce hatch rate. Overall, exposure of tarnished plant bug adults to novaluron, regardless of adult age or exposure route, reduced egg viability. However, the impact on oviposition rate and net reproductive rate varied with adult age and exposure route. This understanding of sublethal impacts of novaluron, in addition to lethal impacts on nymphs, should be considered when choosing application times to maximize effects on *L. lineolaris* populations.

**Introduction**

Tarnished plant bug, *Lygus lineolaris* Palisot de Beauvois (Hemiptera: Miridae), is the most economically important pest of cotton *Gossypium hirsutum* Linnaeus (Malvales: Malvaceae) in the Midsouthern United States (Williams 2012). It has a host range of over 700 plant species, including agronomic crops, wild hosts, fruits, and vegetables (Young 1986, Parys 2014). Tarnished plant bug populations use available flowering hosts for feeding and reproduction in the spring and move to cotton when these host plants senesce. Tarnished plant bug can feed on all stages of the cotton plant, from vegetative stages through early lint development of the last harvestable bolls. Feeding generally causes abscission of small squares and bolls, ultimately leading to yield losses (Russell 1999, Layton 2000). Multiple insecticide applications are made each year to control tarnished plant bug cotton in the Midsouthern region (Cook 2017), and resistance to several classes of insecticides has developed (Snodgrass 1996, Snodgrass and Scott 2000, Snodgrass et al. 2009, Parys et al. 2017). One of the insecticides currently used for tarnished plant bug management is novaluron, an insect growth regulator that disrupts chitin synthesis, a mode of action typical of benzoylphenyl urea (Retnakaran et al. 1985). This chemistry is active on immature stages of insects spanning Lepidoptera, Coleoptera, Diptera, and Hemiptera (Ishaaya et al. 1996). The benzoylphenyl urea’s are thought to interrupt
the transport of certain proteins required for chitin synthesis (Oberlander and Silhacek 1998). In susceptible insects, the endocuticle is compromised after exposure to novaluron, leading to disruption of ecdysis and eventual mortality in the immature stages. Numerous studies report that novaluron has harmful effects on immatures of various species (Xu et al. 2017, Ishaaya and Horowitz 1998, Ishaaya et al. 2003, Maxwell and Fadamiro 2006). These symptoms usually appear during molting, when chitin is being produced and broken down (Verloop and Ferrell 1977), making this product efficacious on nymphs, but not causing direct mortality in adults. Because insect growth regulators fail to kill adults, they are typically mixed with an adulticide when mixed ages of tarnished plant bugs are present (Catchot et al. 2014). While the lethal impact of novaluron on nymphs is known, sublethal effects on other stages are not documented but may also be important to the overall impact of this insecticide on tarnished plant bug management. For example, a single application of novaluron applied to a population of mostly adult *L. lineolaris* suppressed nymph densities for >2 wk., even though adult densities were not reduced (Gore, unpublished data). Another trial showed that maximum yield was preserved when novaluron was applied to immigrating adult populations before nymphs were present (Gore, unpublished data). While this product is known to have a long residual effect on nymphs based on long-term suppression of nymph densities (Gore et al. 2018), it is not known if this suppression is directly from the residues impacting young nymphs, or if it is causing sublethal impacts on the adults. We hypothesized that novaluron is influencing fecundity of adults. Novaluron has been shown to cause sublethal effects on adults such as hatch rate reduction of other insect species like the codling moth *Cydia pomonella* Linnaeus (Lepidoptera: Tortricidae), Colorado potato beetle *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae), and red flour beetle *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae) (Gökçe et al. 2009,
Alyokhin et al. 2008, Wise et al. 2007), so a series of experiments were conducted to quantify the impacts and test the potential sublethal effects of the insect growth regulator novaluron on adult tarnished plant bug.

**Materials and Methods**

**Tarnished Plant Bug Rearing and Maintenance**

In 2005, a laboratory colony of tarnished plant bugs was established at the Mississippi State University Insect Rearing Facility in Starkville, MS. The colony was collected from uncultivated hosts in Mississippi and has had periodic infusions of tarnished plant bug from similar hosts. The colony was maintained as described by Musser et al. (2012) at 27°C, 70% relative humidity with a 16:8 (L: D) cycle. In 2017, the light cycle was changed to 14:10 (L:D). The colony was fed a semisolid oligidic diet (Cohen 2000) which included 33.6 ppm fumagillin (Musser 2012). Tarnished plant bug diet was presented in Parafilm (Pechiney Plastic Packaging, Menasha, WI) packets that were replaced three times per week. Egg packets were made with a 4% carrageenan solution in a Parafilm packet and changed three times per week. All wild populations collected in the regional field trial were reared in the same manner as the laboratory colony for the duration of the experiment.

**Exposure by Ingestion Trials**

In 2009, the laboratory colony of tarnished plant bugs from the Mississippi State University insect rearing facility was used to test the impact of ingestion of novaluron on adult life span, eggs laid per female, and percent egg hatch. A completely randomized design with four treatments and four replicates was used. Each replicate of each treatment was in one rigid 10-cm L × 10-cm W × 5.91-cm H square plastic Ziploc container (591 ml; S.C. Johnson & Son) with 25
newly emerged adult pairs. The top for the container was modified by cutting and removing 50 cm² from the center. The center was then covered with mesh screen to allow airflow and prevent tarnished plant bug escape. Treatments were constant exposure, weekly exposure, a single exposure, and a control that had no exposure to novaluron. Diet packets were either clean (standard diet packets with no insecticide) or treated (standard diet packets with 600 ppm novaluron). All diet packets for the constant exposure treatment containers were treated packets for the duration of the trial. The weekly exposure treatment received a treated diet packet for two successive days followed by clean diet packets for the remainder of each week. The single exposure treatment received a treated diet packet for the first 2 d of the experiment and then received clean diet packets for 3 wk., the remainder of the experiment. The control always received clean diet packets. Tarnished plant bug adults that had eclosed within the last 24 h were used for the experiment, and the trial continued until all adults died. The experiment was conducted under the same environmental conditions as described above for the laboratory colony. Containers were checked daily for mortality, and the sex of dead insects was determined by examining them under a dissecting microscope. Eggs laid on egg packets were counted three times per week. After counting, egg packets were placed in separate containers and examined daily for 10 d to count nymphs and remove them from the container.

A similar study was later conducted using the same laboratory colony to test the impact of novaluron with a single exposure to tarnished plant bug adults by gender. The single 2-d exposure was chosen for this experiment because it had a large permanent impact on the insects and allowed independent exposure to the males and females. Treatments included four gender combinations; treated males + treated females, treated males + untreated females, untreated males + treated females, and untreated males + untreated females (control). Ten pairs of newly
emerged adults were used for each treatment, and each treatment was replicated three times. Newly emerged adults placed in a small container, exposed to CO₂, sexed under a microscope, and separated by gender were placed in separate 10-cm L × 10-cm W × 5.91-cm H plastic square Ziploc containers (591 ml) and received either treated diet (standard diet packets with 600 ppm novaluron) or clean diet (standard diet packets as already described) for 2 d. After 2 d, females and males were paired according to treatment and fed clean diet for the remainder of the trial. Adult mortality, oviposition, and nymph hatch were monitored as described in the previous trial.

Cage Trials

Two different cage trials were designed to evaluate whether tarnished plant bug adult exposure to novaluron from foliar spray residues would cause the same impacts observed in the diet incorporation experiments. A second objective was to evaluate how long after application the insecticide residue had activity on adults. The first cage trial was conducted in the greenhouse, and later a similar cage trial was conducted in a cotton field at the R. R. Foil Plant Science Research Center, Starkville, MS. In both trials, cotton plants were either sprayed with novaluron (Diamond 0.83 EC, ADAMA, Raleigh, NC) at the highest labeled rate of 658 ml/ha (67.3 g a.i./ha) in water or water alone applied at a rate of 140 l/ha. Cotton in the greenhouse and the field was ~1 m tall and contained both flower buds (squares) and small fruit (bolls) when the novaluron applications were made. The greenhouse cotton was grown in pots that were initially fertilized and watered daily as needed and fertilized a total of three times during the trial. The cotton in the field trial was fertilized according to soil test recommendations and watered by furrow irrigation as needed. No insecticides were previously applied to the cotton in either trial. For spraying, the greenhouse pots were arranged in rows to mimic a field arrangement and spray was applied with a pressurized CO₂ backpack sprayer. Prior to the spray application in both
trials, single plant mesh field cages (approx. 1-m diameter × 1.5-m high) were placed around the plants and tied at the base of the plant or as low on the plant as possible with pony-tail holders so that the cage could later be closed above the plant. The mesh cage was kept below the plant during spraying to prevent interference with the spray. After spraying, five male and five female newly eclosed tarnished plant bug adults from the laboratory colony were enclosed in the single plant cages for 48 h. Adults were placed on the plant 1 h after spraying (0 d) and 1, 3, and 5 d after spraying. After the 48-h exposure period, adults were aspirated from the cages and returned to the laboratory where the insects from a common treatment were combined in plastic containers and fed clean diet for the remainder of their life as in the previously described experiments. Tarnished plant bugs from two plant cages (10 pairs) were combined to make a treatment replicate in the greenhouse study, while adults from six plant cages (30 pairs) were combined to make a treatment replicate in the field study. The greenhouse study had three replications and the field study had four replications. Mortality, oviposition rate and hatch rate data were collected as in the previous trials.

**Regional Field Trial**

A field trial was conducted in cotton-production areas of Arkansas, Louisiana, Mississippi, and Tennessee, to test the effects of novaluron on wild adult tarnished plant bug fecundity. In 2012 and 2013, cotton was the host plant used, whereas in 2017, mustard was used as the host plant because larger numbers of *L. lineolaris* were present for collection. Cotton or mustard was planted in two 16 (97 cm) row × 23 m or larger blocks during the normal planting period at each location with at least 50 m between blocks. Each location had a single replication of the trial. At each location, when adult plant bug density exceeded 10/25 sweeps, the treatment block was sprayed with novaluron at 438 ml/ha (43.54 g a.i./ha) in water applied at 94 l/ha (10
GPA) with a tractor mounted sprayer. The control block was not sprayed. One to two days after application, adults were collected from both the treated and control blocks with a standard sweep net, placed in a shipping container and sent to Mississippi State University. All collections were made between 15 May and 25 August. After arrival at Mississippi State University, tarnished plant bugs were held for 72 h on green beans and diet to allow any insects damaged during collection and shipping to die and be discarded before collecting any data. After initial mortality, female numbers ranged from 5 to 44 (mean = 24.4) per treatment per site in 2012, 28–115 (mean = 55.8) per treatment per site in 2013, and 14–112 (mean = 47.5) per treatment per site in 2017. In all years, L. lineolaris were aspirated from the shipping containers and placed in Rubbermaid Servin’ Saver rectangle 8.3 l containers with a modified lid leaving a 2” sealing frame, shredded paper inside and mesh fabric on the top. Mortality, oviposition, and hatch rates were recorded as described in the ‘Exposure by ingestion trial’ for up to 28 d after treatment.

**Egg Trial**

The laboratory colony was used to evaluate if novaluron applied directly to eggs reduced egg hatch. Rearing conditions and oviposition packets were the same as previously described except that three oviposition packets were placed on each of four cages of adults. One oviposition packet from each adult cage containing 1-d old eggs was placed on the ground and sprayed with a pressurized CO₂ backpack sprayer delivering novaluron at 43.54g a.i./ha in water applied at 94 l/ha (10 GPA) (1-d treatment). Two days later, a second oviposition packet from each cage was sprayed in an identical manner (3-d treatment). The third oviposition packet from each cage was not sprayed (control). The number of nymphs hatched from each oviposition packet was recorded daily for 12 d after application.
Statistical Analysis

The impacts of the treatments were measured by evaluating female and male longevity, egg production rate, hatch rate, and net reproductive rate. Adult longevity was measured as the average days’ adults lived after the commencement of the experiment. Oviposition rate was measured as the number of eggs laid per day per living female. Hatch rate is the number of nymphs divided by the number of eggs. Net reproductive rate was calculated as females produced per female over its life or within a week (Heesterbeek 2002) assuming a female: male ratio of 1:1 for nymphs. This jointly reflects the impacts of female longevity, oviposition rate, and hatch rate. Shapiro–Wilk normality tests on raw data or square root transformed data indicated that data were normally distributed, so data for all trials were analyzed with analysis of variance (PROC GLIMMIX, SAS ver. 9.4, SAS Institute, Cary, NC) using the identity link function and the Gaussian distribution. Degrees of freedom were calculated using the Kenward–Roger method. Fisher’s Protected LSD test with $\alpha = 0.05$ was used to separate means. Means and standard errors were determined using PROC MEANS. Field-based trials were analyzed as a randomized complete block design with replication treated as a random effect. Laboratory-based trials used a completely randomized design. When days after treatment were analyzed, data were consolidated into 7-d groups. Weeks after treatment was a repeated (random) factor in all analyses with a first-order autoregressive covariance structure.

Results

Exposure by Ingestion Trials

For adult longevity, there was no impact from the treatments with average longevity of 13.5–16.1 d for males and females of both treatments (Table 3.1). Overall oviposition rates, hatch rates, and net reproductive rates were reduced when exposed to novaluron, regardless of
exposure frequency, and there were no differences among the three exposure levels (Table 3.1). Oviposition rate varied over time \( (F = 14.08; \text{df} = 3, 32; P < 0.0001) \), but the impact of novaluron exposure was consistent over the 4 wk. of oviposition following exposure, even when insects were only exposed once at the beginning of the experiment (week*treatment interaction \( F = 1.00; \text{df} = 9,32; P = 0.46 \) ) (Fig. 3.1). Neither week after exposure nor its interaction with treatment were significant factors for hatch rate or net reproductive rate (Hatch rate: week \( F = 1.37; \text{df} = 3,31; P = 0.27 \): week*treatment interaction \( F = 0.64; \text{df} = 9,31; P = 0.75 \): Net reproductive rate: week \( F = 1.86; \text{df} = 3,32; P = 0.16 \): week*treatment interaction \( F = 1.65; \text{df} = 3,32; P = 0.14 \) ) . In the study comparing exposure by gender, oviposition rate varied by adult age \( (F = 41.04, \text{df} = 3, 20.35, P < 0.0001; \text{Fig 3.2A}) \), but there were no differences between treatments (Table 3.2) nor was there an interaction between treatment and adult age \( (F = 1.47, \text{df} = 9, 20.61, P = 0.224) \). However, treatment impacted hatch rate (Fig. 3.2B), and therefore net reproductive rate (Fig. 3.2C). For both factors, there was also an interaction between treatment and adult age, with the greatest impacts occurring 2–3 wk. after treatment. Overall, exposure of females to novaluron (regardless of male exposure) caused an 85–91% reduction in net reproductive rate, whereas exposure of only males reduced the net reproductive rate by 32% (Table 3.2).

**Cage Trials**

In the green house cage trial, there were no differences between novaluron and control treatments in oviposition rate regardless of the age of the insecticide residue when the adults were exposed (Fig. 3.3A). However, there was a decrease in hatch rate (Fig. 3.3B) from adults placed on novaluron residues within 1 d after application. By 3 d after application, the hatch rate of adults exposed to residues was not affected. The net reproductive rate was only reduced for
adults exposed to novaluron 1 d after application. When analyzing the greenhouse cage data by week after exposure, week was sometimes a significant factor, but there were no interactions of week and treatment (data not shown). In the field cage trial, the 0-d data were discarded due to very low oviposition from control insects. The oviposition, hatch, and net reproductive rates for adults exposed to 1-d-old residues were all reduced compared with the control (Fig. 3.4A–C), whereas there were no treatment differences for adults exposed to 3- and 5-d-old residues (Fig. 3.4A–C). Analyses of the same data by week revealed only one significant treatment by week interaction, namely for net reproductive rate when exposed to residues 1 d after treatment (Fig 3.5).

Regional Field Trial

In the regional field trial conducted in cotton (2012, 2013) and mustard (2017), there was no significant treatment difference in oviposition rate, or net reproductive rate, even though there was a significant reduction in hatch rate (Table 3.3). The number of weeks after exposure was significant for all factors (Fig. 3.6) (Eggs/F/D $F = 5.26, \text{df} = 3, 47.77, P = 0.003$; Hatch rate $F = 3.13, \text{df} = 3, 47.93, P = 0.034$; Net reproductive rate $F = 12.25, \text{df} = 3, 45.49, P < 0.0001$), but there were no interactions between treatment and week (Eggs/F/D $F = 0.61, \text{df} = 3, 47.77, P = 0.612$; Hatch rate $F = 0.13, \text{df} = 3, 47.93, P = 0.944$; Net reproductive rate $F = 0.03, \text{df}=3,44.62, P = 0.992$). There was no replication within a site, so no statistical analysis was possible by site. However, the differences between treatments varied widely between sites. At some sites hatch rate or net reproductive rate in the novaluron treatment was as low as 50% of the control, while there were no differences between treatments at other sites, contributing to large variance and an inability to detect statistical differences between the treatments.
Egg Trial

There were no differences in hatch rate when 1- or 3-d-old eggs were exposed to novaluron compared with an untreated control ($F = 0.02; \text{df} = 2,6; P = 0.98$). Control egg packs had a $33.1 \pm 4.2\%$ hatch rate, 1-d-old, exposed egg packs had a $33.9 \pm 8.8\%$, and 3-d-old, exposed eggs had a $33.2 \pm 8.6\%$ hatch rate.

Discussion

Overall, novaluron exposure to *L. lineolaris* adults reduced egg hatch rate and corresponding nymph production, and this persisted for most of the reproductive life of the tarnished plant bug. These results are consistent with other research on *L. lineolaris* (Catchot et al. 2020) and are similar to observations in adults of Colorado potato beetle, *Leptinotarsa decemlineata*, and house fly, *Culex pipiens* Linnaeus (Diptera: Culicidae) mosquito when exposed to novaluron (Cutler et al. 2005, Alyokhin et al. 2009, Djeghader et al. 2014). Other laboratory research had shown that when *L. lineolaris* adults ingested novaluron 1 d after emergence, oviposition never occurred (Catchot et al. 2020). However, novaluron exposure to older adults (6 and 10 d after emergence) resulted in no reduction in oviposition, but there was still a reduction in hatch rate and nymph production (Catchot et al. 2020). While the current studies show ingestion of novaluron by adults resulted in more pronounced sublethal impacts than contact exposure, contact with dried residues on foliage up to 1 d after application resulted in the same trends, often leading to decreased nymph production. While dried residues only impacted adults exposed within 1 d of application, the depression of nymph populations could persist for several weeks when adult migration is minimal. The depression has normally been considered a result of insecticide residues remaining active on nymphs, but these studies suggest that it may also be due to a reduction in nymphs produced by treated adults. Adults lay most of
their eggs 2–3 wk. after emergence, so a well-timed novaluron application to newly emerged adults could reduce nymph populations through reduced hatch rates for several weeks. Since most mid-season insecticide applications in cotton target primarily nymphs, the suppression of nymph populations from an early novaluron application would lead to fewer mid-season insecticide applications targeting nymphs, reducing both the economic and environmental burden of *L. lineolaris* management.

Laboratory studies show ingested novaluron reduces potential oviposition by newly emerged females through decreasing chitin synthesis in ovarian tissue, thereby disrupting oocyte development (Catchot et al. 2020). The mechanism for reduced hatch rate of eggs laid by exposed females has not yet been determined. Adult males exposed to novaluron also contributed to a lower net reproductive rate, but the mechanism is unclear. Novaluron could impact sperm viability, or the males may transfer some novaluron to females though surface contact or during mating.

As shown in the regional field study when adults of unknown ages were sprayed with novaluron, the impact of novaluron on adults is not great enough or reliable enough to justify it as a standalone insecticide targeting adult *L. lineolaris* population, but it will slightly reduce egg hatch. Therefore, the optimal application timing and the benefit of adding novaluron to another application should consider the sublethal impacts of novaluron on tarnished plant bug adults. In a nonreplicated large plot study where timing of novaluron applications was varied and cotton yield was measured, cotton yield was higher when novaluron was applied when adult numbers were high and nymphs were few, than later when the population was mostly nymphs (Gore, unpublished data). While oviposition rate reduction seems to occur when very young adults are exposed to novaluron (Catchot et al. 2020), the more important reduction in hatch rate, can occur
from exposure to all ages of adults (Catchot et al. 2020). Novaluron exposure is lethal to nymphs, and it reduces the ability of adults to produce nymphs. Thus, the application of novaluron on adult populations can contribute to suppression of *L. lineolaris*, even when nymphs are not present at the time of application.

**Acknowledgments**

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Table 3.1  Mean (SEM) adult tarnished plant bug longevity and productivity after varying frequencies of exposure to 600 ppm novaluron in artificial diet. Means in a column followed by the same letter do not differ significantly (Fisher’s Protected LSD $\alpha = 0.05$).

<table>
<thead>
<tr>
<th>Novaluron Exposure</th>
<th>Male Longevity (d)</th>
<th>Female Longevity (d)</th>
<th>Eggs/Female/d$^a$</th>
<th>% Egg Hatch$^a$</th>
<th>Net Reproductive Rate$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>15.9 (2.47)$^a$</td>
<td>16.1 (1.57)$^a$</td>
<td>6.6 (1.53)$^a$</td>
<td>48 (4.0)$^a$</td>
<td>23.02 (6.71)$^a$</td>
</tr>
<tr>
<td>Once</td>
<td>14.0 (1.83)$^a$</td>
<td>17.2 (1.51)$^a$</td>
<td>2.7 (0.38)$^b$</td>
<td>12 (11)$^b*$</td>
<td>3.18 (3.03)$^b*$</td>
</tr>
<tr>
<td>Weekly</td>
<td>15.4 (2.90)$^a$</td>
<td>15.5 (1.86)$^a$</td>
<td>1.9 (0.34)$^b$</td>
<td>3 (2.0)$^b$</td>
<td>0.29 (0.18)$^b$</td>
</tr>
<tr>
<td>Constant</td>
<td>13.5 (1.05)$^a$</td>
<td>14.3 (0.80)$^a$</td>
<td>1.7 (0.60)$^b$</td>
<td>1 (0.5)$^b$</td>
<td>0.15 (0.06)$^b$</td>
</tr>
</tbody>
</table>

$^a$Statistical analysis conducted on square root transformed data. Untransformed data shown.

* Contains an outlier (as reflected in the large SEM). 3 replicates were similar to the “Constant” treatment, but the other replicate was similar to the “None” treatment.

Table 3.2  Mean (SEM) adult tarnished plant bug productivity after a single 2-day exposure of males and/or females to 600 ppm novaluron in artificial diet. Means in a column followed by the same letter do not differ significantly (Fisher’s Protected LSD $\alpha = 0.05$).

<table>
<thead>
<tr>
<th>Novaluron Exposure</th>
<th>Eggs/Female/day</th>
<th>% Egg Hatch</th>
<th>Net Reproductive Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>8.31 (0.41)$^a$</td>
<td>42 (1.7)$^a$</td>
<td>24.72 (0.66)$^a$</td>
</tr>
<tr>
<td>Females Only</td>
<td>8.42 (0.94)$^a$</td>
<td>6 (0.5)$^c$</td>
<td>3.60 (0.30)$^c$</td>
</tr>
<tr>
<td>Males Only</td>
<td>7.55 (1.04)$^a$</td>
<td>30 (3.4)$^b$</td>
<td>16.84 (3.94)$^b$</td>
</tr>
<tr>
<td>Both Males and Females</td>
<td>9.52 (0.79)$^a$</td>
<td>3 (0.3)$^c$</td>
<td>2.08 (0.22)$^c$</td>
</tr>
</tbody>
</table>

$^a$Statistical analysis conducted on square root transformed data. Untransformed data shown.

* Contains an outlier (as reflected in the large SEM). 3 replicates were similar to the “Constant” treatment, but the other replicate was similar to the “None” treatment.
Table 3.3  Least square mean (SEM) adult tarnished plant bug productivity after being collected from fields sprayed with 43.54-g novaluron a.i./ha or left unsprayed (control) in regional field studies during 2012, 2013, and 2017

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Eggs /Female/day</th>
<th>% Hatch</th>
<th>Net Reproductive Ratea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.09 (0.55)a</td>
<td>35.9 (2.2)a</td>
<td>7.30 (1.60)a</td>
</tr>
<tr>
<td>Novaluron</td>
<td>4.06 (0.55)a</td>
<td>29.1 (2.2)b</td>
<td>5.86 (1.51)a</td>
</tr>
<tr>
<td>F, (df)</td>
<td>0.00, (1,22)</td>
<td>5.00, (1,24)</td>
<td>2.97, (1,14)</td>
</tr>
<tr>
<td>P&gt;F</td>
<td>0.963</td>
<td>0.035</td>
<td>0.1057</td>
</tr>
</tbody>
</table>

aStatistics calculated on square-root transformed data. Untransformed data shown.
Figure 3.1  Mean egg (±SEM) production after exposure of adult *Lygus lineolaris* to novaluron. Exposure was 600 ppm novaluron mixed into a standard diet beginning when adults were 1-d old. The ‘Once’ treatment was exposure for 2 d at the beginning of the trial followed by standard diet for the remainder of the trial. The ‘1/wk.’ treatment was exposure for 2 d every week followed by 5 d on standard diet. Data were square root transformed for statistical analysis. Untransformed data shown.
Figure 3.2  Mean oviposition (A), hatch (B), and nymph (C) production rates (±SEM) per week after exposure of adult Lygus lineolaris to novaluron. Exposure was to 600 ppm novaluron mixed into a standard diet for 2 d beginning when adults were 1-d old followed by standard diet for the remainder of the trial. ‘None’ was fed only standard diet. ‘Treated M’ had males fed novaluron diet, but females were on standard diet. ‘Treated F’ had females fed novaluron diet, but males were on standard diet, and ‘Both’ had both males and females fed on treated diet.
Figure 3.3  Greenhouse cage trial. Comparisons of overall mean (±SEM) oviposition (A), hatch (B), and net reproductive rates (C) after tarnished plant bug adults from a laboratory colony were exposed to 67.3-g novaluron a.i./ha or water (control) dried residues on cotton in a greenhouse beginning 0–5 d after treatment. (A) Pairwise comparisons of eggs/female/day: 0 DAT ($F = 1.70$ df = 1,4; $P = 0.262$), 1 DAT ($F = 0.02$; df = 1,4; $P = 0.887$), 3 DAT ($F = 0.64$; df = 1,4; $P = 0.468$), and 5 DAT ($F = 0.00$; df = 1,4; $P = 0.974$). (B) Pairwise comparisons of % hatch: 0 DAT ($F = 14.60$; df = 1,4; $P = 0.019$), 1 DAT ($F = 55.45$; df = 1,4; $P = 0.002$), 3 DAT ($F = 0.02$; df = 1,3; $P = 0.905$), and 5 DAT ($F = 0.94$; df = 1,4; $P = 0.387$). (C) Pairwise comparisons of net reproductive rates: 0 DAT ($F = 1.69$; df = 1,2; $P = 0.324$), 1 DAT ($F = 14.06$; df = 1,4; $P = 0.020$), 3 DAT ($F = 4.35$; df = 1,2; $P = 0.172$), and 5 DAT ($F = 0.00$; df = 1,4; $P = 0.957$). * denotes that pairwise treatments were significantly different (Fishers Protected LSD at $\alpha = 0.05$).
Figure 3.4  Field cage trial. Comparisons of overall mean (±SEM) oviposition (A), hatch (B), and net reproductive rates (C) after tarnished plant bug adults from a laboratory colony were exposed to 43.5-g novaluron a.i./ha or water (control) dried residues on cotton (Starkville, MS) beginning 0–5 d after treatment. (A) Pairwise comparisons of eggs/female/day: 1 DAT ($F = 9.08; \text{df} = 1,6; P = 0.024$), 3 DAT ($F = 0.68; \text{df} = 1,6; P = 0.441$), and 5 DAT ($F = 0.03; \text{df} = 1,6; P = 0.862$). (B) Pairwise comparisons of % hatch: 1 DAT ($F = 27.84, \text{df} = 1,6, P = 0.002$), 3 DAT ($F = 3.46, \text{df} = 1,6, P = 0.112$), and 5 DAT ($F = 0.16, \text{df} = 1,6, P = 0.704$). (C) Pairwise comparisons of net reproductive rates: 1 DAT ($F = 14.30; \text{df} = 1,6; P = 0.009$), 3 DAT ($F = 1.69; \text{df} = 1,6; P = 0.241$), and 5 DAT ($F = 0.61; \text{df} = 1,6; P = 0.463$). * denotes that pairwise treatments were significantly different (Fishers Protected LSD at $\alpha = 0.05$).
Figure 3.5  Net reproductive rate field cage interaction. Comparison of net reproductive rates (females produced per female) when adults were exposed to either 43.5-g novaluron a.i./ha or water (control) dried residues 1 d after treatment on cotton. The net reproductive rates shown (±SEM) are the weekly contributions to the overall net reproductive rates for each treatment. The interaction of treatment and week was significant ($F = 4.06; df = 3, 18.05; P = 0.0227$) (Fishers Protected LSD at $\alpha=0.05$)
Figure 3.6  Open field response to novaluron application. Comparisons of weekly mean (±SEM) oviposition (A), hatch (B) and net reproductive rates (C) of tarnished plant bug adults collected from hosts 1 d after a spray application of 43.5-g novaluron a.i./ha or collected from a nearby unsprayed area.
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CHAPTER IV

NOVALURON PREVENTS OOGENESIS AND OVIPOSITION BY INDUCING
ULTRASTRUCTURAL CHANGES IN OVARIAN TISSUE OF YOUNG
ADULT TARNISHED PLANT BUG


Abstract

The tarnished plant bug, Lygus lineolaris (Palisot de Beauvois), has emerged as a major pest of cotton, Gossypium hirsutum L, in the mid-southern USA. In the early 1990s L. lineolaris populations developed resistance to several classes of conventional insecticides, increasing the need for insecticides with alternative modes of action such as insect growth regulators (IGRs) for integrated pest management (IPM). The benzoylphenyl urea (BPU) class of IGRs acts by disrupting the growth and development of immature stages of insects, but little is known about its impact on adult stages. The effect of novaluron (Diamond™ 0.83EC), a BPU with known chitin synthesis inhibitor activity, was investigated on adult females of L. lineolaris. Treatment of 1-day-old adults with 600 ppm of novaluron in the diet prevented oviposition, while treatment of older females had no impact on oviposition. Oral novaluron exposure of adults of all ages reduced the viability of eggs laid. Novaluron treatment caused ultrastructural changes in the ovaries of 1-day-old adults (48 h post exposure), distorting the follicular epithelial cell
architecture of developing oocytes. Additionally, novaluron treatment decreased the chitin content in ovarian tissue. Our results suggest that chitin or chitin-like components in the developing ovaries of adult *L. lineolaris* are a target of IGRs such as novaluron, but its activity is specific to a critical time during development. This enhances our understanding of the effects of BPUs on adult insects and could lead to incorporation of IGRs in IPM for controlling adult insect pest populations in the field.

**Introduction**

The tarnished plant bug, *Lygus lineolaris* (Palisot de Beauvois), is the most important insect pest of cotton in Arkansas, Louisiana, Mississippi, Missouri, and Tennessee (Mid-South) (Williams 2016, Cook 2018). *Lygus lineolaris* is polyphagous and documented to have the broadest feeding niche of any known arthropod (Robbins et al. 2000). More than 385 plant species in 55 families across North America have been reported to be hosts of *L. lineolaris* (Young 1986). On cotton, most of the damage from *L. lineolaris* occurs during the period from the onset of squaring (flower buds) through the blooming period, but this pest can feed at any growth stage of the plant (Layton 2000). Damage caused by *L. lineolaris* feeding results in abscission of squares and bolls, leading to loss in yield (Layton 1995, Russell 1995). Foliar-applied insecticides are often used to manage infestations of *L. lineolaris*. The impact of *L. lineolaris* is amplified by its ability to become resistant to insecticides, making management of the tarnished plant bug more difficult (Snodgrass 1996, Snodgrass and Scott 2000, Snodgrass et al. 2009, Parys et al. 2017). As a result, insecticides are often rotated or tank mixed to help maintain effective control (Catchot et al. 2014).
The availability and use of biorational insecticides is a valuable insect pest management option for growers and pest management practitioners. The current suite of biorational insecticides include insect growth regulators (IGRs), characterized by biological activity interfering with specific developmental processes of insects. Among these are chitin synthesis inhibitors represented largely by benzoylphenyl urea’s (BPUs). Efforts to synthesize optimal analogs of BPUs followed their characterization as an insecticide class with a unique chemistry and mode of action (Retnakran et al. 1985). Novaluron, (±)-1-[3-chloro-4-(1,1,2-trifluoro-2-trifluoro-methoxyethoxy) phenyl]-3-(2,6-difluorobenzoyl) urea, one such analog, with several formulations currently marketed for use on field crops, ornamentals, fruits, and vegetables, is a potent acylurea (Ishaaya and Horowitz, 1998). Novaluron has activity against numerous insect species of the orders Lepidoptera, Coleoptera, Hemiptera, and Diptera (Ishaaya et al. 1996). No studies have specifically examined the mode of action of novaluron, but the general mechanisms and effects of other BPUs apply. The mode of action of BPUs on immature stages of insects has largely been attributed to inhibition of chitin biosynthesis causing interference with cuticle formation (Ishaaya et al. 1996). However, BPUs do not readily block chitin synthesis in cell-free systems, nor are they reported to block chitin biosynthetic pathways in intact larvae (Oberlander and Silhacek, 1998). While the precise biochemical explanation of the insecticidal activity of BPUs has not been completely defined, the most likely hypothesis is that they interrupt in vivo synthesis and/or transport of specific proteins required for assemblage of polymeric chitin (Oberlander and Silhacek, 1998). At the organismal level, symptoms are usually expressed at molt when chitin is being actively produced and broken down (Verloop and Ferrell 1977). The integrity of the endocuticle is thus compromised, resulting in disruption of ecdysis and eventual death in the juvenile stages (Retnakaran et al. 1985). In general, only immature stages have been
reported to be affected, and all effects, including complete molt inhibition, malformed pupae, and failure to feed, are a consequence of malformation of cuticle or decreased chitin content (Retnakaran and Wright 1987, Xu et al. 2017).

The detrimental effects of novaluron on immature stages (larvae or nymphs) of various insect species have been documented (Ishaaya et al. 1996, Xu et al. 2017, Ishaaya et al. 1998, Ishaaya et al. 2003, Maxwell and Fadamiro 2006, Parys et al. 2016). In addition, some reports have shown that adult exposure to novaluron through ingestion or contact can have an effect in some insects, for example reduced oviposition and hatching rates in newly emerged adults of *Leptinotarsa decemlineata*, Colorado potato beetle (Cutler et al. 2005, Alyokin et al. 2009) reduced egg hatching in *Cydia pomonella*, codling moth (Kim et al. 2011), and decreased oviposition and hatching in *Culex pipiens*, mosquito (Djeghader et al. 2014). Moreover, lufenuron, another BPU, has been shown to impact oogenesis in *Aedes aegypti* and *Rhodnius prolixus* (Moreira et al. 2007, Mansur et al. 2010). This suggests that insect growth regulators such as novaluron may be used to target adults of *L. lineolaris*, curbing their ability to reproduce. To test this hypothesis, we investigated the effects of novaluron treatment on newly emerged and older adults of *L. lineolaris* and documented its effects on oviposition, egg hatching, ovarian ultrastructure development and maturation, and chitin content in carcass and ovarian tissue.

**Materials and Methods**

**Insect Rearing**

A colony of tarnished plant bugs established in 2005 at Mississippi State University was utilized for this study. This colony was collected from uncultivated hosts in Mississippi and had periodic infusions of wild tarnished plant bugs numerous times since establishment. The colony
was reared as described by Musser and Knighten (2012) and maintained at 27 °C, 70% relative humidity with a 14:10 L:D cycle. The colony was fed a semi-solid oligidic diet (Cohen 2000) that also included 33.6 ppm fumagillin (Musser and Knighten 2012). Diet was provided in Parafilm (Pechiney Plastic Packaging, Menasha, WI, USA) packets and changed three times per week. Egg packets were made using autoclaved carrageenan (Gelcarin, FMC BioPolymer, Philadelphia, PA, USA) (20 g) and water (500 mL). Fifty milliliters of the carrageenan solution were poured into parafilm packets (10 × 20 cm cut, folded, and sealed to make a packet) to provide a flat egg substrate. Egg packets were changed three times per week.

**Experimental Procedure**

The experiment used a randomized complete block design with a factorial arrangement of two factors [L. lineolaris age (1, 6, or 10 days after eclosion) and novaluron exposure (yes or no)] replicated three times. Freshly eclosed (within 24 h) adults of L. lineolaris were separated by sex. Ten males and ten females were randomly selected to be placed together in a plastic Ziploc container (591 mL) (S.C. Johnson & Son, Racine, WI, USA) with shredded paper inside, with a mesh screen and modified Ziploc top with the center cut out leaving a 3 cm rim (Fig. 4.1). A container with 20 L. lineolaris adults was the experimental unit for this trial and there were six containers per replicate. Novaluron (Diamond 0.83EC, ADAMA, Raleigh, NC, USA) was incorporated into the diet for 2 days (48 h) beginning when they were 1-, 6-, or 10-days post eclosion. For incorporation of novaluron into the diet, 1 L of the diet described above was prepared and poured into an autoclaved bottle, to which was added 600 μL of novaluron. The diet was mixed by shaking for 30 s and 50 mL was dispensed into each Parafilm diet pack. The control diet was the same but without novaluron. Diet was placed in each L. lineolaris container within 24 h of being prepared and kept in the container for 48 h. The concentration of novaluron
used (600 ppm) was chosen because it is within the recommended concentration of applying Diamond insecticide to control *L. lineolaris* in 93 L/ha (10 gal/ac) as per the product label (Anonymous 2015, Catchot 2017). After 48 h, the diet packs were replaced with fresh diet without novaluron three times a week for the duration of the study. The environmental conditions during the bioassays were the same as described in the insect rearing section.

**Oviposition and Hatching**

For determining the oviposition rate, egg packets were placed on the rearing container along with diet packets three times per week throughout the period of the experiment (up to 31 days). When changing packets, containers were also examined for any dead insects. Dead insects were removed from the container and sexed in order to adjust the oviposition rate for the number of females present in the container. Egg packets were visually examined under a dissecting microscope to count the number of eggs. After counting, packets were individually placed in containers and covered using chiffon fabric and a modified lid to allow air flow and prevent escape until nymph emergence. Hatched nymphs were counted and removed from the containers three times per week. Most eggs hatched 5–10 days after oviposition, so egg packets were destroyed after 14 days and all remaining eggs were assumed to be unviable.

**Histology of Ovarian Tissue**

In addition to the insects used in the experiment described above, additional female *L. lineolaris* were subjected to the six treatments described in the experimental procedure section. After exposure for 48 h, the females were immediately anesthetized on ice and dissected in cold 0.65% NaCl to remove the ovaries. A total of 30 insects at each age group (untreated and treated separately) in three independent replicates with 10 individuals in each replicate were dissected.
Dissected ovarian tissues were fixed in 4% paraformaldehyde, embedded in HistoGel™ (Thermo Scientific, Waltham, MA, USA) and processed overnight in an Excelsior ES Tissue Processor (Thermo Scientific) with alcohol, xylene, and paraffin. Paraffin-embedded tissue sections (4 μm) were stained with hematoxylin–eosin. Images were acquired using a Zeiss Axio Observer Z1 Inverted Microscope (×20, scale bar = 50 μm; Carl Zeiss Microscopy LLC, Thornwood, NY, USA).

**Chitin Content Estimation**

Chitin content was measured in 1-day-old treated adults, 6-day-old treated adults, 10-day-old treated adults. (48 h post exposure) both in female carcasses (the whole insect without gut, ovaries or fat body) and in dissected ovaries separately from novaluron-treated and untreated diet. The method described by Farnesi *et al* (2015) was used to quantify chitin. Carcass or ovarian tissues from 10 insects within a treatment were homogenized separately in liquid nitrogen and suspended in deionized water. The suspension was centrifuged at 21 000 g for 5 min at 4 °C and the pellet was resuspended in 3% (v/v) sodium dodecyl sulphate (SDS) and incubated at 100 °C for 15 min. Samples were centrifuged again at 21 000 g for 5 min at 4 °C and pellets washed with deionized water and resuspended in 2.1 M KOH with heat treatment at 130 °C for 1 h. To the suspension was added 75% ethanol (2.5× volume of original suspension) and it was kept on ice for 15 min. To this mixture was added 60 μL of Celite 545 (Millipore Sigma #22140) suspension (supernatant of 1 g of suspension in 12.5 mL of 75% ethanol). Samples were centrifuged at 15 000 g for 10 min and pellets were washed with 40% ethanol and then with deionized water. The final pellet (insoluble chitosan) was frozen and stored at −20 °C until the assay was performed. The pellet was resuspended in 100 μL of deionized water to which 100 μL of 5% NaNO₂ and 100 μL of 5% KHSO₄ were added and incubated at room temperature (RT).
for 15 min. Parallel controls were run with just deionized water instead of sample pellets. The samples were vortexed and centrifuged at 1500 g for 5 min at 4 °C. Two parallel replicates of 150 μL each were combined separately with 1/3 volume of 12.5% ammonium sulfamate and 1/3 volume of freshly prepared 12.5% 3-methyl-benzo-2-thiazolone (MBTH) (Millipore Sigma #129739). Samples were vortexed and incubated at 100 °C for 5 min. Following cooling to RT, 50 μL of 0.83% FeCl$_3$.6H$_2$O was added to the samples, and they were vortexed and incubated at RT for 25 min. Aliquots of 200 μL were transferred to 96-well microplates and absorbance at 650 nm was measured in a Biotek Synergy H1M UV–visible microplate reader. Chitin amount was expressed in glucosamine units according to a standard curve obtained with commercial d-(+)-glucosamine hydrochloride (Millipore Sigma #G4875).

**Data Analysis**

Bioassay data on oviposition, hatching, and nymph emergence are represented as mean ± SD of three independent replicates. One-way ANOVA (Kruskal–Wallis test) with Dunn’s post-test was used to analyze differences in the total number of eggs laid during lifetime (mean ± SD), oviposition rate, and hatching (Fig. 4.2, middle panel of Fig. 4.3, and left panel of Fig. 4.4). Unpaired t-test with Welch’s correction was used for analyzing differences in oviposition rate and hatching (10 days treated adults) (right panel of Fig. 4.3 and left panel of Fig. 4.4) between untreated and treated groups. The D’Agostino & Pearson omnibus normality test and one-sample t-test was used for analyzing differences in oviposition rate for 1 day treated adults between untreated and treated groups (Fig. 4.3 left panel). Since no eggs were laid by 1 day treated adults throughout their life, there was no hatching or emergence of nymphs compared to similar aged untreated adults, and thus these were not taken into account for statistical calculations. For chitin content analysis (mean ± SEM) between two treatments the
Mann–Whitney t-test was used (Fig. 4.6). All statistical analyses were conducted, and graphs generated using GraphPad Prism v6.0 (San Diego, CA, USA).

**Results**

**Impacts of novaluron on oviposition, hatching, and nymph emergence.**

No differences in mortality of either males or females was observed on exposure to novaluron at any of the ages studied when compared to untreated control. The effects of novaluron were documented on oviposition and the emergence of nymphs. Adult females exposed to novaluron at a concentration of 600 ppm in the oligidic diet beginning at day 1 after eclosion did not lay eggs throughout their lifetime (Figs. 4.2 and 4.3, left panel), whereas there was no difference in the mean number of eggs laid or oviposition rate for adults treated 6 or 10 days after eclosion compared to untreated groups (Figs 4.2 and 4.3). Since exposure of adults 1 day after eclosion resulted in no eggs, there was no emergence of nymphs. Overall, hatch rate was reduced by novaluron exposure of adults treated at 6 or 10 days after eclosion (Fig. 4.4). Hatch rate was rapidly reduced following exposure to novaluron (Fig. 4.4, left panel). Adults exposed at 6 days of age had a reduced hatch of eggs laid during days 7–10 \( P < 0.05 \). By day 11 and continuing for the remainder of the experiment, no eggs hatched from those laid by adults treated on day 6. Similarly, the hatch rate of eggs laid during days 11–31 was reduced for adults treated on day 10 \( P < 0.05 \), Fig. 4.3, right panel). The reduced egg hatch in 6- and 10-day-old adults exposed to novaluron was also reflected in a reduction in the number of nymphs produced.

**Ultrastructural changes in ovarian development and maturation of young adults induced by novaluron**

To unravel the physiological underpinnings of the impact of novaluron on 1-day-old adults, we conducted a histological analysis of ovarian tissue from untreated and treated 1-day-
old adult females (Fig. 4.5). In approximately 23% of the treated individuals that were dissected we observed a complete failure of ovarian development. In the rest of the treated individuals where ovarian tissue was present, we documented several ultrastructural changes in ovarian development. We found that early vitellogenic oocytes in novaluron-treated insects showed degenerated ovarian follicular epithelial cells. In untreated insects, a single layer of follicular epithelial cells was arranged around each oocyte and these follicular cells became rounded and large as the oocyte enlarged towards maturity. Novaluron treatment affected follicular epithelial cells as well as resulting in distorted oocytes (Fig. 4.5(a),(d),(f)). In untreated adults, the telotrophic ovarioles of *L. lineolaris* had nurse cells located in the germarial region and here vitellogenin deposits were found. In ovaries of untreated individuals, a well-developed vitellogenic oocyte (Fig. 4.5(b)) with germarium and vitellarium regions with vitellogenin deposits (Fig. 4.5(c)) was observed. Ovaries from novaluron exposed females showed a distinct reduction of vitellogenin deposits within the germarium and vitellarium regions (Fig. 4.5(e)).

**Novaluron treatment decreases chitin content in ovaries**

Chitin contents were assayed (as represented by glucosamine levels) in insect carcasses and ovarian tissues of 1-day-old adult females (untreated and treated). Treated 6- and 10-day-old adults were not sampled for chitin content since we did not observe any effect of novaluron treatment on ovarian development at these ages. While no significant difference in the chitin contents of insect carcasses was observed, a 45% decline in chitin content in ovarian tissues of 1-day-old adult females exposed to novaluron was recorded (Fig. 4.6).
Discussion

In this study, the effects of novaluron on oviposition, egg hatching, ovarian ultrastructure, and chitin content were evaluated in adult females of *L. lineolaris*. While BPUs are the most commonly used chitin synthesis inhibitors (Liu et al. 2019), the exact mode of action is not fully understood, as chitin biosynthesis is a complex process. A genome editing study using the CRISPR/Cas9 technique in *Drosophila melanogaster* suggests that BPUs may directly target the chitin synthase (*CHS1*) gene (Douris et al. 2016). This would require demonstration in *L. lineolaris*. There are documented ovicidal and larvicidal toxicities in many arthropod pest species from various orders, including Coleoptera, Diptera, Hemiptera, Lepidoptera, and Acari (reviewed by Merzendorfer (Merzendorfer 2013)). Interestingly, we did not observe any differences in mortality of either males or females from ingestion of novaluron at any of the ages studied when compared to untreated controls, in agreement with no effects on males and females fed with diflubenzuron or lufenuron as reported earlier in *Drosophila* (Gangishetti et al. 2009). However, an effect of novaluron was observed in both young and old adults. In the 1 day treated adult cohort, novaluron treatment resulted in no oviposition, whereas novaluron treatment of older adults reduced egg hatching. Among the treated individuals in the 1 day treated adult group, approximately 23% showed no ovarian tissue development at all, whereas the rest (about 77%) of the individuals showed ultrastructural changes (degenerated follicular epithelial cells, distorted oocytes, or reduced vitellogenin deposits) in ovarian tissues. However, novaluron did not reduce oviposition in older adults (6 or 10 days old) but did impact egg hatching and nymph emergence. This was similar to findings that treating adults (one or both sexes) of codling moth, *Cydia pomonella*, with novaluron (both ingestion and contact) did not impact the number of eggs laid but reduced their hatch rate (Kim et al. 2011). Kim *et al.* (2011) also showed that novaluron
did not have any direct toxicity (contact or ingestion) on the adults. An increase in fecundity of young beetles of *L. decemlineata* on exposure (foliage treatment) to novaluron was reported by Alyokhin *et al.* (2009). They attributed their findings to a hormetic response. However, young beetles did not lay viable eggs when feeding was initiated on treated foliage from adult eclosion, and they suggested that this could be because the insects were not able to become reproductively mature (Alyokhin *et al.* 2009, Alyokhin and Ferro 1999). In the present study, we document that novaluron treatment of older adults (6 days or 10 days) affects egg hatching, reducing nymph production, which reduces population growth.

We hypothesize that the effects of novaluron on blocking oviposition in young adults and decreased egg hatching in older adults could be due to its ability to inhibit chitin biosynthesis. Chitin is a linear polymer composed of β (1 → 4) linked *N*-acetyl-β-d-glucosamine (GlcNAc, 2-acetamido-2-deoxy-d-glucopyranose) (Roberts 1992). Chitin biosynthesis in living organisms takes place in three steps. In the first step, chitin synthase promotes the polymerization of GlcNAc in the presence of divalent cations (e.g., Mg$^{2+}$) as cofactors, which forms the polymer chain. In the second step, the native chitin chain is translocated across the membrane and released into the extracellular space. In the third step, the chitin polymer chains are assembled to form crystalline microfibrils/nanocrystals (Merzendorfer 2006). Subsequently these nanocrystals cluster into chitin–protein fiber, creating a network of chitin fibrils with interspaces filled with pigments, nano-sized inorganic compounds, and other substances (Merzendorfer 2006). In insects, polysaccharide chitin is present in the larval, pupal, and adult integument, and in the peritrophic matrix of the midgut (Gangishetti *et al.* 2009, Moussian *et al.* 2005, Arakane *et al.* 2005, Merzendorfer and Zimoch 2003). In addition, chitin has been detected in the serosal
cuticle of the mosquitoes *Aedes hexodontus*, *Ae. aegypti*, *Anopheles gambiae*, and the beetle *Troblium castaneum* (Beckel 1958, Rezende et al. 2008, Goltsev et al. 2009, Jacobs et al. 2013). It was also reported that a chitin-like component is present in eggshells, eggs and ovaries of *Ae. Aegypti*, and the serosal cuticle of both *An. aquasalis* and *Culex quinquefasciatus* (Moreira et al. 2007, Catchot 2017). The observed effects of novaluron on egg hatching may be due to disruption of the chitin biosynthesis pathway, which may be required for chitin incorporation into eggshells (Moreira et al. 2007, Catchot 2017). Interruption of chitin incorporation may impact normal egg development and the ability to hatch. It has been suggested that the follicular epithelium of the developing oocyte in ovarian tissue might have an important role in the chitin synthesis process (Mansur et al. 2010), as it is known to be an active tissue in the synthesis and transport of yolk and nonyolk proteins to the developing oocytes (Hubner and Anderson 1972, Ma and Ramaswamy 1987). Vitellogenins are glycoproteins that are synthesized in the fat body and then transported and sequestered into the developing egg (Swevers et al. 2005). They are transported to the ovaries via the hemolymph and provide nutrients to the developing oocyte. Disruption of the follicular epithelium by novaluron could specifically affect vitellogenin deposition and hence reduce nutritional supplies to developing oocyte. This could result in cessation of the process of oogenesis, as seen when 1-day-old adults were exposed to novaluron. A decrease in the number of eggs laid with a reduction of viability of eggs on lufenuron and triflumuron treatment has been reported in *Rhodnius prolixus* (Mansur et al. 2009, Henriques et al. 2016). Interestingly, we did not observe any reduction in oviposition or ultrastructural changes in ovarian tissue when 6- or 10-day-old adults were exposed to novaluron. A reduction in egg viability, however, was noted by exposure of 6- or 10-day-old adults to novaluron. Oral exposure to triflumuron has been shown to decrease chitin contents in mated and non-mated
adults of *R. prolixus* (Henriques et al. 2016). A marked dose and age-dependent decrease in incorporation of [3H]-GlcNAc in the ovaries of *R. prolixus* has also been shown (Mansur 2010). These studies, corroborated by this current study, indicate that BPUs could affect chitin incorporation in adults exposed to BPUs and could also affect chitin biosynthesis in ovarian tissue.

**Conclusion**

Chitin is a critical component of not only the arthropod cuticular exoskeleton and peritrophic matrix of the gut but can also be an important component of developing ovaries and eggshells. Many chemicals have been developed to target chitin biosynthesis, among which BPUs are the oldest and the best known (Liu et L. 2019). The general application of BPUs can reduce chitin biosynthesis, leading to hatching defects and abortive molting in immature insects of various insect orders. However, much less is known of its effects on adult insects. In this study we demonstrated that novaluron, a BPU, can impact oviposition by affecting ovarian maturation in *L. lineolaris* and hatching by a yet unclear mechanism. The effect on oviposition is very specific to the age at which the insect is exposed to the IGR. Egg viability, on the other hand, was uniformly affected regardless of the age of the treated adults. Further studies are underway looking at relevant routes of exposure (ingestion or contact or both) and its relevance at the field level and aiming to elucidate the precise mechanism by which novaluron inhibits chitin biosynthesis and egg viability.

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Figure 4.1 Representative images of rearing containers. (a) Rearing container showing cut-away lid replaced with a mesh top with shredded paper inside with *L. lineolaris* adults. (b) Rearing container with diet pack placed on top of mesh.
Figure 4.2  Effect of novaluron treatment on total eggs oviposited by *L. lineolaris*. Mean number of eggs laid (±SD) during lifetime when exposed to 600 ppm of novaluron at various adult ages. One-day-old adults when treated with novaluron did not oviposit throughout their lifetime. Columns with the same letter are not significantly different (Kruskal–Wallis test, $P < 0.05$).
Figure 4.3  Effect of novaluron on number of eggs laid ($\pm$SD) per female of *L. lineolaris* per day. Treatment of 1-day-old adults (left) resulted in no oviposition at 4–6 days (unpaired *t*-test with Welch’s correction for 1-day-old adults at $P < 0.05$) sampling compared to untreated adults and throughout their lifetime. Treatment of 6-day-old adults (middle) and 10-day-old adults (right) with novaluron showed no impact on oviposition (Kruskal–Wallis test at $P < 0.05$ for 6-day-old adults and unpaired *t*-test with Welch’s correction for 10-day-old adults at $P < 0.05$).
Figure 4.4 Effect of novaluron on percentage of eggs hatched (±SD) when exposed to 600 ppm of novaluron at various adult ages of *L. lineolaris*. On treatment with novaluron, eggs laid by 6-day-old adults (left) showed marked reduction in egg hatching compared to parallel untreated controls at 7–11 days sampling and no egg hatching at 11–31 days. Different superscripts are significantly different (Kruskal–Wallis test, \( P < 0.05 \)). Eggs laid by 10-day-old adults treated with novaluron (right) showed significantly reduced hatching (unpaired *t*-test with Welch’s correction at \( P < 0.05 \)).
Figure 4.5  Representative photomicrographs of histological changes in ovaries of *L. lineolaris* following treatment with novaluron. (a)–(c) Normal ovarian follicles of untreated control insect showing (a) normal early vitellogenic oocyte (EvO) with distinct follicular epithelial cells (FC), (b) well-developed vitellogenic oocyte (VO), (c) ovarian follicle with vitellogenic oocyte (VO) showing distinct germarium (G) and vitellerium (V) regions. *Denotes vitellogenin deposits. (d)–(f) Ovarian follicles of insects treated with novaluron (d) distorted oocyte (DO) (e) vitellogenic oocyte (VO) with large spaces/vacuoles (Sp) in germarium/vitellerium interphase. *Denotes diminished vitellogenin deposits (f) degenerated follicular epithelial cells (Dfc). Scale bar = 50 μm.
Figure 4.6  Chitin content in (a) carcass and (b) ovarian tissue of *L. lineolaris* exposed to novaluron. Data are represented as mean ± SEM (*n* = 10). Mann–Whitney test revealed a significant (*P* = 0.0015) decline in chitin content (represented by glucosamine levels) in ovarian tissue of 1-day-old adults exposed to novaluron compared to untreated control ovarian tissue. No difference was observed in the chitin content of the insect carcass (*P* = 0.7959) on exposure to novaluron.
References


APPENDIX A

GOOD FIT ASSAYS USED IN ANALYSIS FOR YEAR, REGION, AND COMPOUNDS
TESTED FOR RESISTANCE MOTORING OF TARNISHED PLANT BUG
POPULATIONS
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APPENDIX B

NOVALURON PREVENTS OOGENESIS AND OVIPOSITION BY INDUCING
ULTRASTRUCTURAL CHANGES IN OVARIAN TISSUE OF YOUNG
TARNISHED PLANT BUG ADULTS
Figure B.1  Mortality (± SD) over 13 d in *L. lineolaris* adults exposed to a single exposure of 600 ppm of novaluron (Diamond™ 0.83EC) for 48 h in diet at 1, 6, or 10 d after emergence. No differences in mortality were recorded in either males or females.
Figure B.2  Impact of novaluron treatment on nymph emergence (number of nymphs (± SD) per female per day) in *L. lineolaris*. Nymphal emergence from eggs of 6 d treated adults (left) showed marked reduction at 7-11 d of sampling compared to parallel untreated controls and no emergence at 11-31 d. Different superscripts are significantly different (Kruskal-Wallis test P<0.05). Nymph emergence from eggs laid by 10 d adults treated with novaluron (right) showed significantly reduced hatching (unpaired t-test with Welch’s correction at P<0.05).
Figure B.3  Impact of novaluron treatment on nymph emergence (number of nymphs (± SD) per female per day) in *L. lineolaris*. Nymphal emergence from eggs of 6 d treated adults (left) showed marked reduction at 7-11 d of sampling compared to parallel untreated controls and no emergence at 11-31 d. Different superscripts are significantly different (Kruskal-Wallis test P<0.05). Nymph emergence from eggs laid by 10 d adults treated with novaluron (right) showed significantly reduced hatching (unpaired t-test with Welch’s correction at P<0.05).