Comparisons of *Lygus lineolaris* populations from the Mississippi Delta and Hills agricultural regions

By

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Lygus lineolaris (Palisot de Beauvois) is a pest of cotton (Gossypium hirsutum L.) in Mississippi, particularly in the Delta region of the state. It is estimated that 3.1-fold more insecticide applications are needed to control this pest and it costs producers 3.6-fold more in the Delta region than in the Hills region. These differences have been attributed to increased insecticide resistance in the Delta and the possibility of a biotype of L. lineolaris in the Delta that is better adapted to cotton than the population in the Hills. A series of experiments were undertaken to evaluate these differences. The first experiment compared developmental time, survival, fecundity, hatch rate, and the cox1 region of the genome of L. lineolaris populations, from both regions, reared on several diets. Data did not reveal any differences in the region of origin but showed that diet had a significant impact on all variables measured except hatch rate. Comparisons of the cox1 region revealed that two cox1 clades exist in the Delta while only one clade exists in the Hills. The second experiment compared the polygalacturonase (PG) system of L. lineolaris from each region. No differences in PG gene expression or enzyme activity,
based on the region of *L. lineolaris* collection, were observed. Specific PG genes that could be a possible target for RNAi insecticides or polygalacturonase-inhibiting proteins in genetically modified cotton were not identified. The third experiment compared LC₅₀s, esterase activity, and glutathione s-transferase (GST) activity of *L. lineolaris* from the two regions. Data revealed that LC₅₀s and mortality were not different in regards to the region of origin; however, activity of GST revealed a strong trend of higher activity in Delta populations while the activity of esterase was significantly higher in Delta populations, indicating increased metabolic resistance in this region. The fourth study used *L. lineolaris* nymphs to compare the abilities of Delta and Hills populations to damage cotton in field cages. Damage was minimal and few differences between regions were recorded. Overall, our data revealed that metabolic insecticide resistance was the only difference between the two regions.
DEDICATION

Dedicated to my wife and children, who were patient with me and supported me through this journey.
ACKNOWLEDGEMENTS

I would first like to thank the ONE TRUE GOD, Jesus Christ, whose mercy and grace toward me made getting this degree possible. I also thank my wife who has been faithful to encourage me and let me do what needed to be done without complaining. I also thank Dr. Musser and Dr. Krishnan whose patience with my thousands of questions made this all possible, as well as the rest of my committee for the support and help they gave me.
## TABLE OF CONTENTS

DEDICATION .................................................................................................................... ii

ACKNOWLEDGEMENTS ............................................................................................... iii

LIST OF TABLES ............................................................................................................ vii

LIST OF FIGURES ........................................................................................................... ix

CHAPTER

I. INTRODUCTION AND LITERATURE REVIEW ......................................................... 1

   Cotton ................................................................................................................... 1
      Production and value .................................................................................... 1
      Economic losses to insect pests ................................................................. 1
   Tarnished Plant Bug .................................................................................... 2
      Association with cotton ................................................................................ 2
      Life cycle ..................................................................................................... 2
      Feeding and damage .................................................................................... 8
   Host plant phenology .................................................................................. 10
   Insecticide resistance .................................................................................. 12
   Economic thresholds .................................................................................... 13
   Population differences .................................................................................. 14
      Mississippi’s cotton growing regions ..................................................... 14
      Mitochondrial DNA ............................................................................. 16
      Biology .................................................................................................... 17
      Phenology ............................................................................................... 18
      Insecticide resistance ........................................................................... 18
   Objectives .................................................................................................... 19
   References .................................................................................................... 21

II. COMPARISONS OF *LYGUS LINEOLARIS* POPULATIONS FROM 
    TWO DISTINCT GEOGRAPHICAL REGIONS OF 
    MISSISSIPPI .................................................................................. 31

   Abstract ............................................................................................................ 31
   Introduction ...................................................................................................... 32
   Materials and methods .................................................................................. 36
      Insect rearing ........................................................................................... 36
III. EXPRESSION AND ACTIVITY OF POLYGALACTURONASE IN LABORATORY REARED AND WILD POPULATIONS OF *LYGUS LINEOLARIS* ........................................56

Abstract ............................................................................................................56
Introduction ......................................................................................................57
Materials and methods .....................................................................................62
  2012 *L. lineolaris* field collections ............................................................62
  Laboratory assays .......................................................................................62
  Sex and age comparisons .....................................................................62
  Nymph and adult comparisons.............................................................63
  2013-2014 field collections ........................................................................63
Gene expression ...............................................................................................64
  Dissection and isolation of salivary glands ............................................64
  RNA isolation ......................................................................................64
  Removal of genomic DNA ..................................................................65
  cDNA synthesis ...................................................................................66
  qRT-PCR..............................................................................................67
Enzyme activity ...............................................................................................67
Statistical analyses ............................................................................................69
Results ..............................................................................................................71
  2012 field collected *L. lineolaris* gene expression .........................71
  Sex and age gene expression comparisons ........................................73
  Nymph and adult gene expression comparisons ................................75
  2013-2014 wild collected *L. lineolaris* gene expression and enzyme activity ..........................................................76
Discussion ........................................................................................................77
Acknowledgements ..........................................................................................82
References .........................................................................................................83

IV. COMPARISONS OF INSECTICIDE SUSCEPTIBILITY AND DETOXIFICATION ENZYMES IN MISSISSIPPI POPULATIONS OF *LYGUS LINEOLARIS* .................................................................87

Abstract ............................................................................................................87
Introduction ......................................................................................................88
Materials and methods .....................................................................................94
Insect rearing ..............................................................................................94
Insecticide assays .......................................................................................95
Glutathione s-transferase activity ..............................................................96
Esterase activity .........................................................................................97
Statistics .....................................................................................................98
Results ............................................................................................................99
Insecticide assays .......................................................................................99
Glutathione s-transferase activity ............................................................101
Esterase activity .......................................................................................102
Discussion ......................................................................................................103
Acknowledgements ........................................................................................108
References ......................................................................................................109

V. A BRIEF NOTE ON THE EFFECTS OF DELTA AND HILLS POPULATIONS OF LYGUS LINEOLARIS NYMPHS CAGED ON COTTON ..............................................114

Abstract ..........................................................................................................114
Introduction ....................................................................................................114
Materials and methods ...................................................................................115
Results ............................................................................................................117
Discussion ......................................................................................................117
Acknowledgments ..........................................................................................119
References ......................................................................................................120

VI. CONCLUSION AND FUTURE CONSIDERATIONS ..................................121

Why is Lygus lineolaris a More Serious Pest in Delta Cotton? ..........121
Future directions ............................................................................................122
LIST OF TABLES

1.1 Summary of the number of days of development of various life stages of *L. lineolaris* in relation to temperature and diet. .................................................4

1.2 Summary of the percentage of *L. lineolaris* nymphs surviving to adult eclosion in relation to temperature and diet. ...........................................5

1.3 Summary of the fecundity of *L. lineolaris* females in relation to temperature, diet, and oviposition substrate. .........................................................6

1.4 Summary of the longevity of adult *L. lineolaris* in relation to temperature, sex and diet .........................................................................................6

1.5 Summary of the percent of *L. lineolaris* nymphs emerging from eggs in relation to diet, oviposition substrate, and temperature. .........................7

1.6 Important wild host species of *L. lineolaris* in the midsouthern United States. ......................................................................................................12

2.1 Mean and standard error (SE) of measurements of *L. lineolaris* developmental time .........................................................................................43

2.2 Mean and standard error (SE) of *L. lineolaris* nymphal survivorship to adult eclosion, fecundity, and the percentage of egg hatch for each diet .................................................................................44

3.1 F-statistics for 2012 wild *L. lineolaris* gene expression ..............................................71

3.2 F-statistics for sex and age comparisons of *L. lineolaris* gene expression ............................................................74

3.3 F-statistics for nymph and adult comparisons of *L. lineolaris* gene expression ..............................................................................................75

3.4 F-statistics for 2013-2014 wild *L. lineolaris* gene expression and enzyme activity. .................................................................................................77

3.5 Mean and standard error (SE) of the enzyme activity of PG in wild *L. lineolaris* collected in Mississippi during 2013-2014. ....................................79
4.1 Probit analyses and LC50 estimates of adult *L. lineolaris* populations in Mississippi during 2014.
LIST OF FIGURES

1.1 Map showing the geographic regions of Mississippi. ........................................15
1.2 Estimated yearly insecticide applications for *L. lineolaris* on cotton in the Delta and Hills regions of Mississippi. .................................................16
2.1 Mississippi geographic regions and locations of *L. lineolaris* collections. ....33
2.2 Maximum likelihood tree of Mississippi *L. lineolaris* and related mirids, mtDNA *cox1*. .................................................................................................46
2.3 Haplotype network of mtDNA *cox1* from Mississippi *L. lineolaris*.........47
3.1 Map showing the geographic regions of Mississippi. .........................................61
3.2 Back-transformed mean and standard error of the effects of host and region on total relative PG gene expression in wild *L. lineolaris* in 2012. .........................................................................................................72
3.3 Back-transformed mean and standard error of the expression of PG genes in wild *L. lineolaris* in 2012. ......................................................................................73
3.4 Back-transformed mean and standard error of the total relative PG gene expression of reared *L. lineolaris* from the study of age and sex.........74
3.5 Back-transformed mean and standard error of the expression of PG genes in reared *L. lineolaris* in the study of age and sex. .................................75
3.6 Back-transformed mean and standard error of the expression of PG genes in *L. lineolaris* in the study of nymphs and adults. ............................76
3.7 Back-transformed mean and standard error of the effects of host on relative PG gene expression in wild collected *L. lineolaris* from Mississippi during 2013-2014. .................................................................78
3.8 Back-transformed mean and standard error of the effects of host on type on relative total PG gene expression in wild *L. lineolaris* collected from Mississippi during 2013-2014.................................................79
4.1 Geographic regions of Mississippi and locations of *L. lineolaris* collections..................................................................................................................93

4.2 Estimated yearly insecticide applications for *L. lineolaris* on cotton in the Delta and Hills regions of Mississippi. .................................................................94

4.3 Mean glutathione s-transferase activity in Mississippi *L. lineolaris* colonies during 2014. ........................................................................................................102

4.4 Mean and standard error of esterase activity in Mississippi *L. lineolaris* colonies during 2014. ............................................................................................103

4.5 Mean and standard error of esterase activity by county in Mississippi *L. lineolaris*. ........................................................................................................104

5.1 Mean and standard error of the percentage of damage and abscission to caged cotton by *L. lineolaris* collected from the Delta and Hills regions of Mississippi during 2014. ........................................118
CHAPTER I
INTRODUCTION AND LITERATURE REVIEW

Cotton

Production and value

Cotton (*Gossypium hirsutum* L.) is one of the most valuable commodities in United States agriculture. The United States is the world’s third largest producer of cotton, planting an average of 3.9 million hectares and producing approximately 3.7 million metric tons yearly (2010-2012) (USDA-NASS 2014). The national value of cotton from 2010 to 2012 was approximately 6.7 billion dollars (USDA-NASS 2014). Mississippi cotton growers planted an average of 205.6 thousand hectares of cotton between 2010 and 2012 with an average yield of 1.0 million bales (USDA-NASS 2014) at a value of approximately 394 million dollars per year (USDA-NASS 2014).

Economic losses to insect pests

One of the biggest challenges to cotton production is controlling insect pests. Managing insect pests cost United States cotton producers an average of 500 million dollars per year from 2012-2014 (Williams 2012-2014). These costs include insecticides, scouting, technology fees, and eradication. Losses from insect feeding reduced yield approximately 2.4% during the same years. During this same period, insect pests cost
Mississippi producers an average of 58.4 million dollars for insect management and losses from insect feeding reduced yield by an average of 8% (Williams 2012-2014).

**Tarnished Plant Bug**

**Association with cotton**

*Lygus lineolaris* (Palisot de Beauvois) (tarnished plant bug) is an important pest of cotton in the United States. It is the predominant cotton feeding mirid species in the Southeast and Midsouth regions of the United States (Layton 2000) and has been listed as a pest in many insect control guides and cotton production guides from universities in the Midsouth and Southeast since at least the 1960s (USDA-ARS 1962, Pack and Tugwell 1976, Baldwin et al. 2010, Stewart et al. 2011, Whitworth et al. 2011, Catchot et al. 2015). Currently, *L. lineolaris* is the most economically important insect pest of cotton in Mississippi (Williams 2012-2014).

**Life cycle**

Crosby and Leonard (1914) describe *L. lineolaris* adults as 1/4 th to 1/5 th inch (approximately 5-6.5 mm) long appearing generally brown with mottling of yellow and reddish browns, Kelton (1975) and Schwartz and Footit (1992) give a similar description. *Lygus* sp. females oviposit eggs in leaf tissue, buds, flower heads, seed stalks and fruit (Taylor 1908, 1909, Chittenden and Marsh 1910, Haseman 1913, Crosby and Leonard 1914), leaving the operculum, which the nymphs emerge through, exposed above the surface of the plant tissue (Cooper and Spurgeon 2011). The eggs are “flask-shaped and obliquely truncate”, 0.95 to 1 mm in length by 0.25 mm wide (Crosby and Leonard 1914). *Lygus lineolaris* nymphs go through five instars and their color ranges
from yellow-green to green to brown. *Lygus lineolaris* nymphs grow from approximately 1 mm in length at eclosion to 4 mm in length at the 5th instar (Crosby and Leonard 1914).

The life cycle of *L. lineolaris* is documented in many research articles, and data from these articles are summarized in Tables 1.1-1.5. Temperature and diet are two of the most important factors in the *L. lineolaris* life cycle, especially for determining the length of time for a generation. Temperature and diet are the primary factors in egg and nymphal developmental time, nymphal survival, fecundity, adult longevity, and hatch rate. Egg incubation time range from 5.2 to 18.4 d, developmental time of first instar nymphs ranges from 2.6 to 8.5 d, second instar development ranges from 2.0 to 6.0 d, third instar development ranges from 1.9 to 6.1 d, fourth instar development ranges from 2.4 to 7.1 d, fifth instar development ranges from 3.4 to 11.7 d, and total developmental time from egg to adult ranges from 12.4 to 48.4 d (Table 1.1). The preoviposition period ranges from 4.9 to 25.4 d (Table 1.1). Based on these data, the generation time of *L. lineolaris* ranges from approximately 22 to 83 d depending upon temperature and diet. Survival of nymphs ranges from 29.0 to 86.7 % (Table 1.2). Fecundity ranges from 0.3 eggs per female per day to 9.5 eggs per female per day (Table 1.3). Longevity of males and females differs. Males survive from 17.3 to 41.2 d while females survive 17.6 to 58.1 d (Table 1.4). Egg hatch ranges from 27.2 % to 82.5 % (Table 1.5).

*Lygus lineolaris* can have multiple, overlapping generations each year (Layton 2000). The number of generations for *L. lineolaris* varies depending upon the geographical location from where the data were collected: two generations in Ontario (Guppy 1958), possibly three generations in New York, Illinois, and some parts of Canada (Knight 1941, Ridgway and Gyrisco 1960b, Stewart and Khoury 1976). Four to
five generations were observed in Missouri (Haseman 1913) and four to five generations are likely in Mississippi as well (Ridgway and Gyrismo 1960a, Bariola 1969). The first one or two generations may be completed on weedy hosts, with the next one to three generations on cotton and the final generation again on weedy hosts (Stadelbacher 1987, Young and Lockley 1990, UC-DANR 1996b).

Table 1.1  Summary of the number of days of development of various life stages of *L. lineolaris* in relation to temperature and diet.

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<td>Phaseolus vulgaris</td>
<td></td>
<td>7.9</td>
<td>5.2</td>
<td>4.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>
Table 1.1 (Continued)

<table>
<thead>
<tr>
<th>Developmental stage</th>
<th>Ref. no.</th>
<th>Diet</th>
<th>Temperature (°C)</th>
<th>Number of days of development</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>16</td>
</tr>
<tr>
<td>Total egg to adult</td>
<td>1</td>
<td>Artificial diet</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Artificial diet</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td><em>Gossypium hirsutum</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td><em>Gossypium hirsutum</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td><em>Gossypium hirsutum</em></td>
<td></td>
<td>48.4</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td><em>Phaseolus vulgaris</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Artificial diet</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Artificial diet</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td><em>Phaseolus vulgaris</em></td>
<td></td>
<td>24.2</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td><em>Phaseolus vulgaris</em></td>
<td></td>
<td>25.4</td>
</tr>
<tr>
<td>Pre-oviposition</td>
<td>2</td>
<td><em>Phaseolus vulgaris</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>period</td>
<td>3</td>
<td><em>Phaseolus vulgaris</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td><em>Apium graveolens</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td><em>Daucus carota</em></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1Reference number: 1) Adams et al. (2014); 2) Ugine (2012); 3) Bariola (1969); 4) Curtis and McCoy (1964); 5) Khattat and Stewart (1977); 6) Ridgway and Gyrisco (1960a).

Table 1.2 Summary of the percentage of *L. lineolaris* nymphs surviving to adult eclosion in relation to temperature and diet.

<table>
<thead>
<tr>
<th>Ref. no.</th>
<th>Diet</th>
<th>Temperature (°C)</th>
<th>Percentage of nymphs surviving</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>16</td>
<td>21</td>
</tr>
<tr>
<td>1</td>
<td>Artificial diet</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Artificial diet</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td><em>Gossypium hirsutum</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td><em>Gossypium hirsutum</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td><em>Phaseolus vulgaris</em></td>
<td>73.0</td>
<td>79.0</td>
</tr>
<tr>
<td>4</td>
<td>Artificial diet</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td><em>Brassica oleracea</em> cv. Italica</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td><em>Zea mays</em>^2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td><em>Zea mays</em>^3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td><em>Phaseolus vulgaris</em></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1Reference number: 1) Adams et al. (2014); 2) Bariola (1969); 3) Curtis and McCoy (1964); 4) Cohen (2000); 5) Abel et al. (2010).

^2R2-3 kernels and cob tips.

^3R1 silks.
Table 1.3  Summary of the fecundity of *L. lineolaris* females in relation to temperature, diet, and oviposition substrate.

<table>
<thead>
<tr>
<th>Ref. no.</th>
<th>Diet</th>
<th>Oviposition substrate</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Artificial diet</td>
<td>gel pack</td>
<td>16</td>
</tr>
<tr>
<td>1</td>
<td>Artificial diet</td>
<td>gel pack</td>
<td>17</td>
</tr>
<tr>
<td>1</td>
<td>Gossypium hirsutum</td>
<td>gel pack</td>
<td>18</td>
</tr>
<tr>
<td>1</td>
<td>Gossypium hirsutum</td>
<td>gel pack</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>Phaseolus vulgaris</td>
<td>gel pack</td>
<td>21</td>
</tr>
<tr>
<td>2</td>
<td>Phaseolus vulgaris</td>
<td>P. vulgaris</td>
<td>22</td>
</tr>
<tr>
<td>3</td>
<td>Phaseolus vulgaris</td>
<td>P. vulgaris</td>
<td>23</td>
</tr>
<tr>
<td>5</td>
<td>Solanum tuberosum</td>
<td>S. tuberosum</td>
<td>24</td>
</tr>
<tr>
<td>5</td>
<td>Phaseolus vulgaris</td>
<td>P. vulgaris</td>
<td>25</td>
</tr>
<tr>
<td>5</td>
<td>Pisum sativum</td>
<td>P. sativum</td>
<td>26</td>
</tr>
<tr>
<td>5</td>
<td>Phaseolus vulgaris</td>
<td>P. vulgaris</td>
<td>27</td>
</tr>
<tr>
<td>5</td>
<td>Apium graveolens</td>
<td>A. graveolens</td>
<td>28</td>
</tr>
<tr>
<td>5</td>
<td>Brassica rapa var. rana</td>
<td>B. rapa var. rana</td>
<td>29</td>
</tr>
<tr>
<td>5</td>
<td>Brassica oleracea cv. Italica</td>
<td>B. oleracea cv. Italica</td>
<td>30</td>
</tr>
<tr>
<td>4</td>
<td>Phaseolus vulgaris</td>
<td>P. vulgaris</td>
<td>31</td>
</tr>
<tr>
<td>4</td>
<td>Apium graveolens</td>
<td>A. graveolens</td>
<td>32</td>
</tr>
<tr>
<td>5</td>
<td>Daucus carota</td>
<td>D. carota</td>
<td></td>
</tr>
</tbody>
</table>

1Reference numbers: 1) Adams et al. (2014); 2) Ugine (2012); 3) Bariola (1969); 4) Khattat and Stewart (1977); 5) Curtis and McCoy (1964).

Table 1.4  Summary of the longevity of adult *L. lineolaris* in relation to temperature, sex and diet.

<table>
<thead>
<tr>
<th>Ref. no.</th>
<th>Diet</th>
<th>Sex</th>
<th>Number of days of from adult eclosion to death</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Phaseolus vulgaris</td>
<td>Female</td>
<td>16</td>
</tr>
<tr>
<td>1</td>
<td>Phaseolus vulgaris</td>
<td>Male</td>
<td>17</td>
</tr>
<tr>
<td>2</td>
<td>Phaseolus vulgaris</td>
<td>Female</td>
<td>18</td>
</tr>
<tr>
<td>2</td>
<td>Phaseolus vulgaris</td>
<td>Male</td>
<td>19</td>
</tr>
<tr>
<td>3</td>
<td>Phaseolus vulgaris</td>
<td>Female</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>Apium graveolens</td>
<td>Female</td>
<td>21</td>
</tr>
<tr>
<td>3</td>
<td>Daucus carota</td>
<td>Female</td>
<td>22</td>
</tr>
<tr>
<td>3</td>
<td>Phaseolus vulgaris</td>
<td>Male</td>
<td>23</td>
</tr>
<tr>
<td>3</td>
<td>Apium graveolens</td>
<td>Male</td>
<td>24</td>
</tr>
<tr>
<td>3</td>
<td>Daucus carota</td>
<td>Male</td>
<td>25</td>
</tr>
</tbody>
</table>

1Reference number: 1) Ugine (2012); 2) Khattat and Stewart (1977); 3) Curtis and McCoy (1964).
Table 1.5 Summary of the percent of *L. lineolaris* nymphs emerging from eggs in relation to diet, oviposition substrate, and temperature.

<table>
<thead>
<tr>
<th>Ref. no.</th>
<th>Host</th>
<th>Oviposition substrate</th>
<th>Percent hatch</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Artificial diet</td>
<td>gel pack</td>
<td>27.2</td>
<td>16</td>
</tr>
<tr>
<td>1</td>
<td>Artificial diet</td>
<td>gel pack</td>
<td>28.9</td>
<td>20</td>
</tr>
<tr>
<td>1</td>
<td><em>Gossypium hirsutum</em></td>
<td>gel pack</td>
<td>36.6</td>
<td>21</td>
</tr>
<tr>
<td>1</td>
<td><em>Gossypium hirsutum</em></td>
<td>gel pack</td>
<td>29.9</td>
<td>22</td>
</tr>
<tr>
<td>2</td>
<td><em>Phaseolus vulgaris</em></td>
<td><em>Phaseolus vulgaris</em></td>
<td>62.0</td>
<td>24</td>
</tr>
<tr>
<td>2</td>
<td><em>Phaseolus vulgaris</em></td>
<td><em>Phaseolus vulgaris</em></td>
<td>78.0</td>
<td>27</td>
</tr>
<tr>
<td>2</td>
<td><em>Phaseolus vulgaris</em></td>
<td><em>Phaseolus vulgaris</em></td>
<td>82.0</td>
<td>32</td>
</tr>
<tr>
<td>3</td>
<td><em>Solanum tuberosum</em></td>
<td><em>Solanum tuberosum</em></td>
<td>82.5</td>
<td>16</td>
</tr>
<tr>
<td>3</td>
<td><em>Phaseolus vulgaris</em></td>
<td><em>Phaseolus vulgaris</em></td>
<td>61.6</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td><em>Pisum sativum</em></td>
<td><em>Pisum sativum</em></td>
<td>74.1</td>
<td>21</td>
</tr>
<tr>
<td>3</td>
<td><em>Apium graveolens</em></td>
<td><em>Apium graveolens</em></td>
<td>55.2</td>
<td>22</td>
</tr>
<tr>
<td>3</td>
<td><em>Brassica rapa var. rapa</em></td>
<td><em>Brassica rapa var. rapa</em></td>
<td>57.0</td>
<td>24</td>
</tr>
<tr>
<td>3</td>
<td><em>Brassica oleracea cv. Italica</em></td>
<td><em>Brassica oleracea cv. Italica</em></td>
<td>41.0</td>
<td>27</td>
</tr>
</tbody>
</table>

1Reference number: 1) Adams et al. (2014); 2) Bariola (1969); 3) Khattat and Stewart (1977).

Generally, overwintering *L. lineolaris* become active during the first warm spring days (Crosby and Leonard 1914). Records of *L. lineolaris* spring activity include: March 20 in NY (Crosby and Leonard 1914), late March in Missouri (Taylor 1908), April 19 in Washington, DC (Chittenden and Marsh 1910), and the end of April in Canada (Kelton 1975, Stewart and Khoury 1976). Snodgrass (2003) showed that diapause was broken in Mississippi Delta populations of *L. lineolaris* at two points. Females overwintering on winter hosts were found with mature eggs by the end of December, while females that overwintered in plant debris did not have mature eggs until the end of January. Eggs were found oviposited in plant tissue as early as the last week of January. The new generation of *L. lineolaris* produced from overwintering females became reproductive at approximately the same day length as when diapause began (12.5:11.5, L:D) (Snodgrass 2003), approximately April 1st.
Feeding and damage

*Lygus lineolaris*, like all hemipterans, have piercing-sucking mouthparts. The piercing-sucking mouthpart is comprised of a bundle of flexible stylets, which consist of paired mandibles and maxillae (Hamer 1972). *L. lineolaris* inserts its stylet bundle into plant tissue, injects saliva containing enzymes, specifically polygalacturonase (PG) to break down plant tissue, specifically pectins, and draws out plant fluids (Hamer 1972, Tingey and Pillemer 1977). The effect of salivary enzymes causes greater injury to plants than the insertion of the stylet into plant tissue, and is primarily due to the enzyme polygalacturonase (PG) (Strong 1970, Girard and Jouanin 1999, Boyd et al. 2002, Shackel et al. 2005, Frati et al. 2006). Research has shown the role of PG is to degrade pectin in the cell wall of plant tissue (Strong and Kruitwagen 1968) by degrading the α-1,4 polygalacturonic acid component of the plant cell wall (De Lorenzo et al. 1997, De Lorenzo et al. 2001, Ridley et al. 2001). The mechanism of PG can be further divided into endo-PG and exo-PG. Endo-PG degrades the α-1, 4 polygalacturonic acid of the cell wall while exo-PG produces monosaccharide galacturonic acid by targeting the non-reducing end of the α-1,4 polygalacturonic acid (De Lorenzo et al. 1997, Ridley et al. 2001). Some forms of PG enzyme may have both endo- and exo-type activity (Cook et al. 1999). Little is known specifically about PG in *L. lineolaris*. Research has shown that polygalacturonase in *L. lineolaris* is regulated by at least three PG genes (PG1, PG2, PG3) (Allen and Mertens 2008) and that the expression of different PG genes may change depending upon diet (Walker and Allen 2010). The PG enzyme has been shown to digest dsRNA (Allen and Walker 2012) and that PG may be important in oviposition site preparation (Cooper and Spurgeon 2011).
Lygus lineolaris feeds on many parts of the cotton plant including: squares, terminals, leaf nectaries, anthers, blooms, and bolls. (Wene and Sheets 1964, Scales and Furr 1968, Pack and Tugwell 1976, Tugwell et al. 1976, Hanny et al. 1977, Tingey and Pillemer 1977, Russell 1999, Layton 2000, Coy et al. 2001, Musser et al. 2009). Feeding by L. lineolaris on cotton results in wilted leaves, burnt leaves, terminal bud destruction, leaf margin necrosis, bloom failure, blasting and shedding of young bolls and squares, reduced plant height, excessive branching, shortened internodes, deformed leaves, non-fertile squares, anther discoloration, dirty lint, and delayed fruiting and maturity (Morrill 1918, Ewing 1929, Cassidy and Barber 1939, Scales and Furr 1968, Bariola 1969, Pack and Tugwell 1976, Tugwell et al. 1976, Hanny et al. 1977, Scott et al. 1985). Feeding on small squares causes extensive internal injury and causes squares to turn brown and abort or to be unfertile (Ewing 1929, Ewing and McGarr 1933, Scales and Furr 1968, Bariola 1969, UC-DANR 1996a). Damage to large squares may result in fruit shed due to lack of pollination (Pack and Tugwell 1976). Wilson (1984) stated that one L. lineolaris can cause two squares to abort per day throughout its life. Squares are most vulnerable to L. lineolaris feeding when less than 5 mm in diameter (UC-DANR 1996a). Feeding on terminals can cause the terminals to abort, leading to a loss of apical dominance and allowing multiple branches to grow, giving the cotton a bushy appearance, known as “crazy cotton” or the “candelabra effect” (Ewing 1929, Pack and Tugwell 1976, Hanny et al. 1977, UC-DANR 1996a). Abortion of terminals also causes cotton to delay fruiting three to fifteen days (Bariola 1969). Delays in fruiting and boll maturity due to L. lineolaris feeding can be especially important in areas where cotton harvest is often adversely affected by weather conditions (Black 1973, Hanny et al. 1977, Wilson 1984,
Coy et al. 2001). Season-long high populations of *L. lineolaris* could reduce cotton yield by possibly 550 kg per ha (Scales and Furr 1968); however, the time between square retention and early bloom is the most susceptible time for cotton (Black 1973, Pack and Tugwell 1976). Adams et al. (2013) showed that early planted cotton and early maturing cotton varieties were less affected by *L. lineolaris*. *Lygus lineolaris* has the potential to be very damaging to cotton yields but the timing of feeding and environmental conditions determine the extent of cotton yield loss.

**Host plant phenology**

*Lygus lineolaris* is a very polyphagous species that has been collected from at least 169 plant species in the MS, AR, LA region (Snodgrass et al. 1984a) and on over 300 species throughout the United States (Young 1986). Hosts include many crops in the United States such as: tree fruit, vegetable crops, forage crops, seed crops, oil crops and fiber crops (Crosby and Leonard 1914, Webster and Spuler 1931, Chandler 1955, Neunzig and Gyrisco 1955, Guppy 1958, Rings 1958, McEwen and Hervey 1960, Davis et al. 1963, Huber and Burbutis 1967, Broersma and Luckmann 1970, Kelton 1975, Hauschild and Parker 1976, Butts and Lamb 1990). When hosts are destroyed or become unattractive as a food source, adult *L. lineolaris* fly to other suitable hosts (Woodside 1947, USDA-ARS 1962), but are generally not attracted to a host until it enters a reproductive growth stage (Fleischer and Gaylor 1987). Weeds typically serve as primary hosts for *L. lineolaris* but if the weeds become unsuitable for feeding they will move to cotton if it is nearby (USDA-ARS 1962). *Lygus lineolaris* may complete one or more generations on wild hosts and the intensity and duration of the movement from wild hosts to cotton depends on the abundance of wild hosts and the availability of flowering
structures on the hosts (Taksdal 1961, Hamer 1972, Pack and Tugwell 1976, Wilson 1984, Fleischer and Gaylor 1988, Layton 2000). Craig (1998) found that understanding the relationship between alternate hosts and cotton is one of the most important aspects to managing \textit{L. lineolaris} in cotton. Cleveland (1982) found that the ratio of \textit{L. lineolaris} in wild hosts compared to cotton was approximately 5:1. Control of early season wild hosts robs \textit{L. lineolaris} of nursery hosts and affects their population movements to cotton (Snodgrass et al. 2000a, Snodgrass et al. 2000b, Snodgrass et al. 2003b, Snodgrass et al. 2003a, Snodgrass et al. 2005a, Snodgrass et al. 2005b, Snodgrass et al. 2006), and according to Fleischer and Gaylor (1987), when rainfall was sufficient to keep wild hosts succulent through the summer, weeds served as a trap crop for \textit{L. lineolaris}. However, during dry years, weeds served as an early season nursery crop that allowed \textit{L. lineolaris} populations to build up then move into cotton during the summer when the weed hosts senesce. Table 1.6 shows 23 of the important wild host plants for \textit{L. lineolaris} in the Midsouth. Fleabane (\textit{Erigeron} spp.) (Tugwell et al. 1976) and horseweed (\textit{Conyza canadensis} (L.) Cronquist) (Latson et al. 1977) have been cited as some of the most important hosts of \textit{L. lineolaris}. Pigweed (\textit{Amaranthus} spp.) has become a more important host in recent years with the increasing presence of glyphosate-resistant populations. Glyphosate resistant weeds are entomologically important because many are also hosts of \textit{L. lineolaris}. Increased weed populations due to glyphosate resistance could lead to increased \textit{L. lineolaris} populations. Table 1.6 denotes (*) glyphosate resistant weeds found in Mississippi and neighboring states according to Heap (2015).
Table 1.6 Important wild host species of *L. lineolaris* in the midsouthern United States.

<table>
<thead>
<tr>
<th>Season&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Species</th>
<th>Common name</th>
<th>Source&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>OW-early</td>
<td><em>Capsella bursa-pastoris</em> (L.) Medik.</td>
<td>Shepherd's purse</td>
<td>1, 7, 10</td>
</tr>
<tr>
<td>OW-early</td>
<td><em>Geranium dissectum</em> L.</td>
<td>Cutleaf geranium</td>
<td>5, 6, 10</td>
</tr>
<tr>
<td>OW-early</td>
<td><em>Lamium amplexicaule</em> L.</td>
<td>Henbit</td>
<td>6, 7, 10</td>
</tr>
<tr>
<td>Early</td>
<td><em>G. carolinianum</em> L.</td>
<td>Carolina geranium</td>
<td>5, 6, 7, 10</td>
</tr>
<tr>
<td>Early</td>
<td><em>Lolium multiflorum</em> Lam.*</td>
<td>Annual/Italian ryegrass</td>
<td>11</td>
</tr>
<tr>
<td>Early</td>
<td><em>Medicago arabica</em> (L.) Huds.</td>
<td>Spotted burclover</td>
<td>4, 7, 10</td>
</tr>
<tr>
<td>Early</td>
<td><em>Oenothera</em> spp.</td>
<td>Primrose</td>
<td>7, 10</td>
</tr>
<tr>
<td>Early</td>
<td><em>Trifolium incarnatum</em> L.</td>
<td>Crimson clover</td>
<td>3, 4, 7</td>
</tr>
<tr>
<td>Early-mid</td>
<td><em>Anthemis cotula</em> L.</td>
<td>Mayweed</td>
<td>3, 4, 5, 7, 10</td>
</tr>
<tr>
<td>Early-mid</td>
<td><em>Erigeron</em> spp.</td>
<td>Fleabane</td>
<td>3, 4, 6, 7, 10</td>
</tr>
<tr>
<td>Early-mid</td>
<td><em>Rumex crispus</em> L.</td>
<td>Dock</td>
<td>3, 4, 7, 10</td>
</tr>
<tr>
<td>Early-mid</td>
<td><em>Vicia</em> spp.</td>
<td>Vetch</td>
<td>5, 7, 9, 10</td>
</tr>
<tr>
<td>Mid</td>
<td><em>Coreopsis tinctoria</em> Nutt.</td>
<td>Tickseed</td>
<td>7, 10</td>
</tr>
<tr>
<td>Mid</td>
<td><em>Torilis arvensis</em> (Huds.)</td>
<td>Hedge-parsley</td>
<td>3, 10</td>
</tr>
<tr>
<td>Mid-late</td>
<td><em>Amaranthus</em> spp.*</td>
<td>Pigweed</td>
<td>4, 7</td>
</tr>
<tr>
<td>Mid-late</td>
<td><em>Ambrosia artemisiaefolia</em> L.*</td>
<td>Ragweed</td>
<td>3, 4, 7, 10</td>
</tr>
<tr>
<td>Mid-late</td>
<td><em>Coryza canadensis</em> (L.) Cronquist*</td>
<td>Horseweed</td>
<td>3, 4, 5, 7, 8, 9, 10</td>
</tr>
<tr>
<td>Mid-late</td>
<td><em>Crotot capitatus</em> Michx.</td>
<td>Wooly croton</td>
<td>4, 10</td>
</tr>
<tr>
<td>Mid-late</td>
<td><em>Helenium amarum</em> (Raf.) H. Rock</td>
<td>Bitterweed</td>
<td>3, 4, 7, 10</td>
</tr>
<tr>
<td>Mid-late</td>
<td><em>Polygonum pensylvanicum</em> L.</td>
<td>Pinkweed</td>
<td>4, 10</td>
</tr>
<tr>
<td>Late</td>
<td><em>Aster</em> spp.</td>
<td>Aster</td>
<td>2, 7, 10</td>
</tr>
<tr>
<td>Late</td>
<td><em>Chenopodium album</em> L.</td>
<td>Common lambsquarters</td>
<td>7, 10</td>
</tr>
<tr>
<td>Late</td>
<td><em>Solidago</em> spp.</td>
<td>Goldenrod</td>
<td>3, 10</td>
</tr>
</tbody>
</table>

* denotes species with populations discovered to be resistant to glyphosate herbicide

1 Stadelbacher (1987); 2, Young and Lockley (1990); 3, Tugwell et al. (1976); 4, Cleveland (1982); 5, Fleischer and Gaylor (1987); 6, Robbins et al. (2000); 7, Snodgrass et al. (1984b); 8, Latson (1974); 9, USDA-ARS (1977); 10, Snodgrass et al. (1984a); 11, Snodgrass et al. (2000a).

2 OW= cited as overwintering host, early= serves as host until approximately June, mid= serves as host from approximately June to September, late= serves as host from about September to overwintering.

**Insecticide resistance**

The importance of *L. lineolaris* as a pest increased with the eradication of the boll weevil, the adoption of transgenic *Bt* cotton, and ultimately with the discovery of

**Economic thresholds**

*Lygus lineolaris* can be very difficult to control with chemical insecticides in areas where insecticide resistance has developed; however, chemical insecticides are still the most efficient method for managing *L. lineolaris* populations. Current insecticide application thresholds for *L. lineolaris* in Mississippi are divided into two categories: first two weeks of squaring and third week of squaring to bloom. *Lygus lineolaris* populations can be measured by drop cloth, visual counts, and sweep net sampling. During the first two weeks of squaring thresholds are met when 1 bug/1.8 m is found on a drop cloth, 5 bugs/100 terminals can be found visually, or 8 bugs/100 sweeps are collected. Starting at
third week of squaring, *L. lineolaris* is not as damaging, thresholds are increased to 3 bugs/1.8 m found on a drop cloth, 10 bugs/100 terminals can be visually counted, or 15 bugs/100 sweeps are collected. Another threshold that can be used from the third week of squaring to bloom is visual observation of dirty squares (squares with discolored yellow buds). A ten percent or higher level of dirty squares indicates an economically damaging level of *L. lineolaris* is present (Catchot et al. 2015).

**Population differences**

*Mississippi’s cotton growing regions*

Mississippi has two important cotton growing regions, the Delta and the Hills (Figure 1.1). The Delta is the flood plain of the Mississippi and Yazoo rivers that extends, in Mississippi, from approximately Vicksburg to the Tennessee state line in western Mississippi. The Delta is an intensively farmed region consisting of 55% row-crop agriculture land, 2% forested land, 35% pasture or otherwise open land, and 7% urban land (Fry et al. 2011). The Hills region is the rest of the state, but the primary cotton production region of the Hills is the north and east part of the state. The primary cotton growing areas of the Hills consists of 20% row-crop agriculture land, 45% forested land, 30% pasture and otherwise open land, and 5% urban land (Fry et al. 2011). Since 1986, the cotton crop losses (Williams 1986-2014) have been reported separately for these two regions of Mississippi, and based on the estimates for each region, *L. lineolaris* have required approximately 2.6-fold more insecticide applications in the Delta than in the Hills region (Figure 1.2).
Figure 1.1  Map showing the geographic regions of Mississippi.

The Yazoo basin is the area referred to as the Delta. The region referred to as the Hills is the rest of the state, but concerning cotton production is primarily the Flatwoods, Pontotoc Ridge, Black Prairie, North Central Hills, and Tombigbee Hills geographic regions, as these are the primary cotton production areas in the Hills region. Source: Hutson (2008).
Figure 1.2 Estimated yearly insecticide applications for *L. lineolaris* on cotton in the Delta and Hills regions of Mississippi.

Trend line equations: Delta 1986 to 1999, no. of applications= -193.4+0.098(year); Delta 2000 to 2014, no. of applications= -595.4+0.299(year); Hills 1986 to 2001, no. of applications= 106.5-0.053(year); Hills 2002 to 2014, no. of applications= -416.7+0.208(year). Data taken from Williams (1986-2014) and piece-wise regression equations estimated using Proc NLIN in SAS 9.3.

**Mitochondrial DNA**

Mitochondrial DNA of *L. lineolaris* populations has been studied. DNA barcoding of mtDNA cytochrome oxidase region (*cox*) of *L. lineolaris* has demonstrated substantial genetic variation and some broad scale geographic diversity in *L. lineolaris* (Burange et al., 2012). Two clades of the *cox*1 region have been discovered and both clades are widely sympatric in North America. Clade 1 is more common in northern (colder) regions, whereas clade 2 is more common in southern (warmer) regions. If
differences in phenology and behavior of *L. lineolaris* populations have arisen between the Hills and Delta regions, then genetic evidence may support such isolation.

**Biology**

Recent research has indicated that biological differences between *L. lineolaris* populations exist. Adams et al. (2014) reported the number of days for *L. lineolaris* nymphal development on cotton to be 1.67 d longer for two colonies collected from the Hills than for the two colonies collected from the Delta. Differences were not seen in developmental time for those fed artificial diet. Differences between cotton and artificial diet may indicate a host performance shift in some populations. Additionally, females from colonies collected from the Delta laid 1.09 more eggs per day than females from the Hills. Taken together, these data indicate that some populations of *L. lineolaris* may be more specialized for reproduction on cotton. These types of host shifts have been documented in other insect pest species. For example, two “strains” of *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae) exist. One strain, the corn strain, prefers corn (*Zea mays* L.), sorghum (*Sorghum bicolor* (L.) Moench), and cotton, while the rice strain prefers rice (*Oryza sativa* L.) and wild grasses (Pashley 1986). These strains showed preference to hosts, poorer performance on less preferred hosts, and differing levels of mixed function oxidases, but both strains can occur in the same habitats (Pashley et al. 1995, Veenstra et al. 1995). Additionally, Karpinski et al. (2014) showed that *Heliothis virescens* Fabricius (Lepidoptera: Noctuidae) strains developed on diets differently, preference for oviposition hosts, and that variation in larval performance had a genetic basis. Both genetic and nongenetic factors can affect specialization of local populations. Similar host preference shifts and genetic divergence could be occurring in
*L. lineolaris* populations leading some to prefer cotton over other hosts. If regional *L. lineolaris* populations have adapted to the prominent host plants in a region, then behavior and biology similar to that seen in *S. frugiperda* and *H. virescens* may be observed.

**Phenology**

If adaptation to host plants has occurred, then host plant phenology may affect *L. lineolaris* damage to cotton. Phenology of *L. lineolaris* populations and available wild hosts has been determined by Cleveland (1982), Fleischer and Gaylor (1987), and Snodgrass et al. (1984b). Additionally Abel et al. (2010) and Snodgrass et al. (2010) determined that corn and soybeans (*Glycine max* (L.) Merr.) were suitable *L. lineolaris* hosts and could serve as a source of populations that can invade cotton. Phenological differences of host plants and *L. lineolaris* populations has not been compared between regions or landscape type; however, Carver et al. (1985) showed that host source of a *L. lineolaris* population had an effect on plant height and fruiting in cotton. This may indicate that movement of *L. lineolaris* populations from some host plant species may have a more serious impact on cotton than movement from other host plant species. However, the results may be caused from differences between populations of *L. lineolaris* independent of previous host plant. In either case, this experiment shows a dissimilarity of *L. lineolaris* populations to adaptation for cotton.

**Insecticide resistance**

Only one study comparing insecticide resistance in Delta and Hills populations is known to have been published. Snodgrass et al. (2009) compared populations of *L.
*lineolaris* from both regions from 2005 to 2007. The populations from the Hills were primarily from the northern portions of the Loess Bluff and North Central Plateau geographical regions (Figure 1.1). They found in 2005, the average LC$_{50}$ for acephate in Hills populations were higher than the Delta populations. Also in 2005, both regions had populations with resistance ratios for acephate of >3.0, which indicates the population may not be manageable with labeled rates of organophosphate insecticides. However, in 2006 and 2007 the average LC$_{50}$s and resistance ratios were higher in Delta populations. They also found that percent mortality in *L. lineolaris* treated with permethrin was lower in Delta collected populations. These data indicate that LC$_{50}$s, resistance ratios, and mortality vary in both Delta and Hills populations of *L. lineolaris*, but, overall, *L. lineolaris* from the Delta tend to be more tolerant of insecticides.

**Objectives**

The economic, biological, and physiological differences observed between the Delta and Hills populations of *L. lineolaris* are not well understood. This research will attempt to evaluate these differences through biological, genetic, and ecological comparisons. The objectives are 1) to determine if regional biological differences exist and if diet is a factor in the differences, 2) to determine if regional differences exist for the mtDNA *cox* clades, 3) to determine if the polygalacturonase system and response to host plants differs among regional populations, 4) to determine if insecticide LC$_{50}$s and levels of detoxification enzymes differ between regional populations, and 5) to make comparisons of damage to cotton between regional populations. The results of these experiments will help rule out or give further evidence for the causes of dissimilar impact to cotton of *L. lineolaris* populations, provide new information about *L. lineolaris*
biology, ecology, and genetics, as well as potentially reveal new IPM strategies for *L. lineolaris*. 
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CHAPTER II

COMPARISONS OF *LYGUS LINEOLARIS* POPULATIONS FROM TWO DISTINCT GEOGRAPHICAL REGIONS OF MISSISSIPPI

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Abstract

The tarnished plant bug, *Lygus lineolaris* (Palisot de Beauvois) (Hemiptera: Miridae) is a major pest of cotton (*Gossypium hirsutum* L.) in the state of Mississippi. Economic data indicate that *L. lineolaris* is a more serious pest of cotton in the Delta region of Mississippi than in the Hills region; however, little data exist comparing the two populations. Two experiments were undertaken to compare *L. lineolaris* from these two geographically distinct regions. In the first experiment, colonies of *L. lineolaris* from each region were reared in the laboratory under controlled conditions and measurements of developmental time, survivorship, fecundity, and hatch rate were compared. The geographic region of origin had no effect on any of the variables measured; however, the diet used for rearing had a significant effect on all variables except hatch rate. In the second experiment, part of the *cox1* gene of the *L. lineolaris* mitochondrial genome was compared between the two populations to examine possible genetic differences between
L. lineolaris from the two regions of Mississippi. Data revealed two cox1 clades in the Delta region and only one cox1 clade in the Hills region. Taken together, the data do not explain the reason for the differences in the severity of damage to cotton in the two regions.

Introduction

Lygus lineolaris (Palisot de Beauvois) (Hemiptera: Miridae) has become a major pest of cotton (Gossypium hirsutum L.) in Mississippi. Since 1979, it has frequently been considered one of the three most costly insect pests of Mississippi cotton (Williams 1997), and since 2003, L. lineolaris was the target of 4.1 insecticide applications per ha per year with an average yearly cost of $110 per ha (Williams 1979-2012). Estimated yield losses and cost of control from L. lineolaris damage are not uniform across the state. Since 1986, the cotton insect losses of Mississippi have been separated into two regions, the Delta and the Hills. The Delta is the region of western Mississippi that is the flood plain of the Mississippi and Yazoo rivers and includes the area west of the Loess Bluffs (Figure 2.1). The Hills region, referred to in the cotton insect losses of Williams (1979-2012) beginning in 1986, is the remainder of the state (Figure 2.1). The Delta region is intensively farmed and has less non-cultivated areas than the Hills region which typically has smaller fields with adjacent non-cultivated areas. The number of insecticide applications targeting L. lineolaris in the Delta region has been estimated to be 3.1-fold greater than in the Hills region, and the costs of control plus the value of the cotton lost per hectare to L. lineolaris has been estimated to be approximately 3.7-fold greater in the Delta than in the Hills (Williams 1979-2012).
Figure 2.1   Mississippi geographic regions and locations of *L. lineolaris* collections.

The insects collected from the Delta region are from the Delta (Alluvial Plain) region and insects collected from the Hills are from other regions of the state. Circled county names indicate counties in which *L. lineolaris* were collected to start colonies. Counties where collections were made for cox1 comparisons are denoted with a star.
The basis for differences in biology and economic impact are uncertain. Biological differences between these regional populations of \textit{L. lineolaris} have previously been reported. Adams et al. (2014) reported the number of days for \textit{L. lineolaris} development to adulthood when fed cotton to be 1.67 d longer for colonies collected from the Hills than for those collected from the Delta. Differences in developmental time were not seen for those fed artificial diet. Differences between populations on some diets may indicate that populations have adapted to different diets in different regions. Additionally, females from colonies collected from the Delta laid 1.09 more eggs per day than females from colonies collected from the Hills. These two factors indicate that some populations of \textit{L. lineolaris} may be more specialized for reproduction on cotton or other plant hosts and that diet impacts \textit{L. lineolaris} biology.

Research has shown that host plant preference and adaptation exist in populations of some species. For example, two “strains” of \textit{Spodoptera frugiperda} (Smith) (Lepidoptera: Noctuidae) exist. One strain, the corn strain, prefers corn (\textit{Zea mays} L.), sorghum (\textit{Sorghum bicolor} L.) and cotton, while the rice-strain prefers rice (\textit{Oryza sativa} L.) and wild grasses (Pashley 1986). These strains showed preference to hosts, poorer performance on less preferred hosts, and differing levels of mixed function oxidases, even though both strains occur in the same habitats (Pashley et al. 1995, Veenstra et al. 1995). Additionally, Karpinski et al. (2014) showed that \textit{Heliothis virescens} F. (Lepidoptera: Noctuidae) strains developed on diets differently, showed preference for oviposition hosts, and that variation in larval performance had a genetic basis. Genetic and non-genetic factors can affect specialization of local populations. Similar host preferences and genetic divergence may be possible in \textit{L. lineolaris} populations, leading
some to prefer cotton over other hosts. If regional *L. lineolaris* populations have adapted to the prominent host plants in a region, then behavior similar to those seen in *S. frugiperda* and *H. virescens* may be expected.

If adaptation to host plants has occurred, then host plant phenology may affect *L. lineolaris* damage to cotton. Phenology of *L. lineolaris* populations and available wild hosts has been determined by Cleveland (1982), Fleischer and Gaylor (1987), and Snodgrass et al. (1984). Additionally, Abel et al. (2010) and Snodgrass et al. (2010) determined that corn (*Zea mays* L.) and soybeans (*Glycine max* (L.) Merr.) were suitable *L. lineolaris* hosts and could serve as a source of *L. lineolaris* that would invade cotton. The phenological differences of host plants and *L. lineolaris* populations has not been compared between regions or landscape types. However, Carver et al. (1985) showed that the host source of *L. lineolaris* populations had an effect on plant height and fruiting in cotton. This may indicate that movement of *L. lineolaris* populations from some host plant species may have a more serious impact on cotton than movement from other host plant species. However, the results may be from differences between populations of *L. lineolaris* independent of previous host plant. In either case, this experiment showed a dissimilarity of adaptation of *L. lineolaris* populations to cotton.

Genetic markers have been useful in comparing populations of organisms. Mitochondrial DNA has proven to be a useful tool for examining both intra-specific and inter-specific genetic diversity in insects. DNA barcoding of mtDNA cytochrome oxidase subunit 1 (*cox1*) has been successful, and has been used to define nearly 400 species of Heteroptera (Park et al. 2011). Various mtDNA sequences have demonstrated substantial genetic variation and some broad scale geographic diversity in *L. lineolaris* (Burange et
al., 2012). If differences in phenology and behavior of *L. lineolaris* populations have arisen between the Hills and Delta regions, then genetic evidence may support such isolation.

Based on the economic, biological, and potential genetic evidence mentioned and the lack of knowledge about why regional differences occur, experiments were performed to evaluate Mississippi *L. lineolaris* populations reared on several diets to further examine regional differences between diets and populations. Finding differences between region and diet would indicate a potential biotype or sibling species shift. Comparisons of mtDNA were performed to determine if genetic evidence supported the idea of phenological isolation.

**Materials and methods**

**Insect rearing**

Colonies (three from the Delta and three from the Hills) of *L. lineolaris* used in the experiment were collected from wild host plants, primarily pigweed (*Amaranthus* sp.), horseweed (*Conyza canadensis* (L.) Cronquist), fleabane (*Erigeron* sp.), and crimson clover (*Trifolium incarnatum* L.) in May and June 2013. Insects were collected with sweep nets, placed into plastic containers, and aspirated into cricket cages for transport back to the Mississippi State University Insect Rearing Center. Insects were then emptied into 8.3 liter plastic containers filled with shredded paper. Containers were equipped with self-sealing lids modified with the center of the lid removed. A fine mesh screen was placed on the containers and held down with the remaining portion of the self-sealing lid. Screen covers and shredded paper were replaced weekly to reduce contamination from fecal material. Insect colonies were kept in a rearing chamber
maintained at 27 ± 2°C at a relative humidity of 65 ± 5% with a 16:8 light: dark cycle. Colonies were reared 2-3 generations until enough insects were available for the experiment. The insects were fed an oligidic artificial diet similar to that of Cohen (2000), presented in a 5 x 5 cm Parafilm (Beemis Company Inc., Neenah, WI) packet placed on top of the screens. Oviposition packets consisting of a 4% carrageenan solution presented in a 2.5 x 2.5 cm Parafilm packet were also placed on top of the screens as this provides a place for the females to oviposit. Oviposition packets containing eggs were transferred three times weekly into 8.3 liter plastic containers.

**Diet experiments**

First instar nymphs from a colony were gently shaken into a cup, counted and transferred into 3.1 liter plastic containers (developmental containers) containing shredded paper and covered with a fine mesh screen held down with a self-sealing lid. Approximately 60 nymphs were used for each replicate of each treatment for each colony. Developmental containers were placed in a rearing chamber under the same conditions as the parent colonies. The food was placed inside each container on top of the shredded paper. The food and amounts placed in each container were as follows: one 3 x 5 cm oligidic artificial diet packet, two broccoli (*Brassica oleracea* L.) florets (approximately 30 g each), one corn ear tip (approximately 5 cm of the apical end of a husked corn ear with along with any silks present) and one corn ear cross-section (approximately 1.5 cm thick with husk), fifteen pinhead to midpoint cotton squares with bracts intact, six to eight green bean (*Phaseolus vulgaris* L.) pods (approximately 10-12 cm long), and five pigweed (*Amaranthus retroflexus* L. and *Amaranthus palmeri* S. Wats.) seed heads (both male and female) (approximately 10-20 cm long) in water picks.
Each diet, with the exception of artificial diet, was replicated within a colony four times. The quantity of diet available was always in excess of food needs, so there was no attempt to make food availability equal. All diets could not be tested at the same time, so the artificial diet treatment with four replicates was used as the common treatment whenever any other diet type was tested. Total replicates of artificial diet for any colony ranged from four to sixteen. Broccoli and green beans were purchased at local grocery stores. To reduce the risks of insecticide residues, broccoli and green beans were soaked in a sodium hypochlorite solution for ten minutes and triple rinsed before being used in the experiment. After washing, broccoli and green beans were placed under a hood to dry. Corn and cotton were from non-\textit{Bt} varieties grown in a field on the Mississippi State University R.R. Foil Plant Science Research Farm. No insecticides were used near these plants. The pigweed was grown in Washington County, MS on a USDA-ARS farm. Pigweed seed heads were cut semi-weekly, placed into water picks, and transported to Mississippi State University. All foods were stored in a refrigerator maintained at approximately 4° C, and any plant tissue not used within one week was discarded. To reduce high nymphal mortality observed in preliminary research, artificial diet was placed in all containers until the nymphs reached third instar. Upon development to third instar, only the plant tissue was available to the insects. Artificial diet, broccoli, corn, and pigweed were replaced three times per week. Green beans were checked daily for mold and replaced as needed. Otherwise they were also replaced three times per week. Cotton squares were replaced daily. Disturbance was minimized in the containers by not removing plant tissue until nymphs had moved to fresher tissue. The progression of development was observed daily and the date was recorded when approximately half of
the nymphs in a container had reached a new instar or adulthood. The number of insects that survived to adulthood was used to calculate survivorship.

The adults that eclosed in the developmental containers were transferred into new containers (fecundity containers) of a similar type and size as the developmental containers. Newly eclosed adults from all replicates of a treatment were combined into one replicate each time adults were available so that an average of 40 adults was in each container. These insects were transferred from the developmental containers three times per week as adults developed. Three to four replicates of fecundity containers were set up for each colony and diet. Diets for this part of the experiment were the same as the developmental phase but placement of some of the diets changed. Artificial diet, green beans, corn ear cross-sections and broccoli were placed on top of the screen while pigweed and cotton squares were still placed inside the containers. A 2.5 x 2.5 cm Parafilm oviposition pack containing 4% carrageenan gel was placed on top of the screen and offered for oviposition. Foods and oviposition packs were replaced three times per week. Both gel packs and plant tissue were examined for eggs. Plant tissue was examined under a stereo microscope and eggs were counted. The plant tissue was discarded due to desiccation and mold that made the eggs on plant tissue less viable. Eggs on gel packs were counted without magnification. Fecundity was measured for approximately 20 d after the first eggs were laid. Dead adults were removed from fecundity containers three times per week and stored in a freezer until they could be sexed and counted.

Oviposition packets containing at least ten eggs were placed into a variety of 0.5-0.75 L plastic containers (hatch containers) with lids and screens similar to those mentioned previously. The number of hatched nymphs was recorded 10 d after the
Oviposition packs were placed into the hatch containers. Oviposition packs that showed signs of fungal growth before the data were recorded were discarded.

Statistical analyses were performed in SAS 9.3 using the Proc Mixed procedure (SAS Institute Inc., Cary, NC). Replicates within a colony were treated as subsamples. Thus, colonies were considered replicates of the region and were used as random factors. Initial analysis revealed that the two colonies collected in 2012 behaved differently than those collected in 2013, so the data for the colonies collected in 2012 were discarded because there was only one colony from each region. Initial analysis also revealed that there was no region x diet interaction (p > 0.2) or main effect of region (p > 0.1) for any variable measured, thus region was considered a random factor for all biological experiments. Degrees of freedom were calculated using the Kenward-Roger method (Kenward and Roger 1997). Differences were considered significant at α=0.05 (Fisher’s LSD).

**DNA analysis**

*Lygus lineolaris* used for DNA comparison were collected from wild hosts from 13 locations in the Delta and Hills regions. Insects were collected in a sweep net and aspirated into cricket cages for transport back to the laboratory. Cricket cages were placed into a -20° C freezer to kill the insects. Ten insects were then removed from the cages and placed into a 70% ethanol solution and shipped to the USDA-ARS Biosciences Research Laboratory, Fargo, ND for testing. Specimens of three other mirids (*Taylorilygus apicalis* (Fieber), *Polymerus basilis* (Reuter), and *Reuteroscopus ornatus* (Reuter)) were also collected for use as outgroups.
DNA was extracted from individuals as described in Burange et al. (2012) and a QIAGEN DNeasy Blood & Tissue Kit (QIAGEN Sciences, Germantown, MD) was used to obtain total genomic DNA. Two mitochondrial regions were selected for amplification. The universal primers LCO (5’-ggtcaacaatactcaataagatgg-3’) and HCO (5’-taaacttcagggtacaaaaatca-3’) amplified a 658 bp segment of cox1, which is the primary insect barcode region. A second pair of primers, ND1-Lyg (5’-tagaattagaagataaccacat-3’) and 16S-Lyg (5’-ttcagaccggcgtgagccagg-3’), were based on the sequence of the complete L. lineolaris mitochondrial genome (Roehrdanz et al. 2014), and these primers yielded a sequence of 480 bp that included a portion of the NADH dehydrogenase 1 gene (ND1), tRNA-Leucine (tRNA-Leu2), and a piece of the 16S ribosomal RNA gene (16S rRNA). This will be referred to as the ND1 region. PCR amplicons were purified using QIAprep Spin Miniprep kit (QIAGEN Inc., Valencia, CA) as per the manufacturer’s protocol. DNA sequencing was carried out by the DNA sequencing facility at Iowa State University, Ames, IA. All of the sequences have been entered in GenBank and labeled with the accession numbers KJ190392-KJ190454 and KJ401345-KJ401413.

DNA sequences were aligned using the Clustal W function of the VectorAdvance software package (Invitrogen Corporation, Carlsbad, California). For estimates of evolutionary divergence the number of base substitutions per site between each pair of sequences were determined and analyses were conducted in MEGA5 (Tamura et al. 2011) using the Maximum Composite Likelihood mode (Tamura et al. 2004). All ambiguous positions were removed for each sequence pair. There were a total of 658 positions in the final dataset for cox1 and 477 nucleotides for ND1. The evolutionary history was inferred in MEGA5 by using the Maximum Likelihood method based on the
Tamura-Nei model (Tamura and Nei 1993). The tree with the highest log likelihood was saved and the percentage of trees in which the associated taxa clustered together is shown next to the branches (bootstrap 100 replicates). Initial trees for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The trees were drawn to scale, with branch lengths measured in the number of substitutions per site. All ambiguous positions were removed for each sequence pair, and separate trees were constructed for cox1 (68 sequences) and ND1 (43 sequences). The cox1 tree was supplemented with sequences from MS Delta insects that were obtained in conjunction with a different study. A ML tree was also prepared using representatives of two L. lineolaris genetic clades in combination with sequences from four other Lygus species and six related mirids, three of which (T. apicalis, R. ornatus, and P. basialis) were collected in Mississippi. The GenBank accessions from this work are KC783526-KC783528, KC783637-KC783643, KC783646-KC783648, KC783654, and KJ411639-KJ411641. A haplotype network of the L. lineolaris cox1 sequences was prepared by hand.

Results

Diet experiments

There was no interaction of region and diet for any of the parameters measured (p>0.2). Furthermore, the region from which the colonies were collected was not a significant main effect for any parameters (p>0.1). Therefore, results are presented for the diets pooled over both regions. Diet had a significant effect on days to 3rd instar (df= 5, 29; F= 5.09, p<0.01), days as 3rd instar (df= 5, 25; F= 7.25; p<0.01), days as 5th instar
(df= 5, 25; F= 3.33; p=0.02), and total days to adult development (df= 5, 25; F= 9.28; 
p<0.01) but not for days as 4th instar (df= 5, 25; F= 1.89; p=0.13). Although artificial 
diet was included in all treatments until third instar, there were significant differences 
between diets in the number of days to third instar (Table 2.1). *Lygus lineolaris* in the 
broccoli treatment developed the quickest, significantly faster than those fed cotton or 
green beans (Table 2.1). Development to third instar on green beans was slowest, and 
was significantly slower than all other diets except cotton. Total developmental time to 
adult eclosion for *L. lineolaris* fed broccoli was faster than for those fed corn, cotton, or 
green bean (Table 2.1). Total developmental time for *L. lineolaris* fed green beans was 
slower than all other diets (Table 2.1). Measurements of developmental time for other 
variables are presented in Table 2.1.

Table 2.1 Mean and standard error (SE) of measurements of *L. lineolaris* 
developmental time.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Days from hatch to third instar Mean (SE)</th>
<th>Days as third instar Mean (SE)</th>
<th>Days as fourth instar Mean (SE)</th>
<th>Days as fifth instar Mean (SE)</th>
<th>Total days from hatch to adult eclosion Mean (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD</td>
<td>5.84 (0.07) B</td>
<td>2.71 (0.12) B</td>
<td>3.13 (0.14) B</td>
<td>4.79 (0.16) C</td>
<td>18.31 (0.19) BC</td>
</tr>
<tr>
<td>Broccoli</td>
<td>5.23 (0.08) C</td>
<td>2.13 (0.06) C</td>
<td>3.13 (0.23) B</td>
<td>4.85 (0.23) C</td>
<td>17.06 (0.52) C</td>
</tr>
<tr>
<td>Corn</td>
<td>5.58 (0.11) BC</td>
<td>3.13 (0.14) AB</td>
<td>3.42 (0.24) AB</td>
<td>5.38 (0.47) BC</td>
<td>19.46 (0.58) B</td>
</tr>
<tr>
<td>Cotton</td>
<td>5.90 (0.17) AB</td>
<td>3.21 (0.12) A</td>
<td>3.04 (0.16) B</td>
<td>5.58 (0.27) ABC</td>
<td>20.03 (0.13) B</td>
</tr>
<tr>
<td>Green beans</td>
<td>6.42 (0.40) A</td>
<td>3.25 (0.22) A</td>
<td>3.71 (0.22) A</td>
<td>6.46 (0.42) A</td>
<td>22.60 (1.20) A</td>
</tr>
<tr>
<td>Pigweed</td>
<td>5.38 (0.15) BC</td>
<td>2.71 (0.25) B</td>
<td>3.17 (0.20) B</td>
<td>5.88 (0.67) AB</td>
<td>18.42 (0.54) BC</td>
</tr>
</tbody>
</table>

Means in a column followed by a common letter are not significantly different according 
to Fisher’s LSD, α=0.05.

Number of observation (N) for all variables measured = 6. An observation is the average 
for a colony.

1artificial diet

†Total days is calculated based on the number of days from hatch to until approximately 
one-half of the nymphs had eclosed as adults. It is not the cumulative number of days of 
each previous developmental measurement.
Diet had a significant effect on nymphal survival (df= 5, 25, F= 21.72, p<0.01) and fecundity (df= 3, 15; F= 6.12; p<0.01), but not on the percent of eggs hatched (df= 3, 12.9; F= 2.5; p=0.11). Nymphs on broccoli, artificial diet, and green beans had significantly higher survival than nymphs reared on corn, cotton, and pigweed (Table 2.2). Nymphs on pigweed had the lowest survival (Table 2.2). Eggs were not found in cotton and pigweed treatments, thus fecundity could only be analyzed for artificial diet, broccoli, corn, and green bean treatments. Fecundity was approximately 1.5-fold higher in *L. lineolaris* fed artificial diet than those fed broccoli and 3-fold higher than in those fed corn (Table 2.2). Fecundity was approximately 2-fold higher in those fed green beans than those fed corn (Table 2.2). The percentage of eggs that hatched ranged from 25.8 to 39.6% (Table 2.2).

<table>
<thead>
<tr>
<th>Diet</th>
<th>Mean (SE)</th>
<th>Mean</th>
<th>N</th>
<th>Mean (SE)</th>
<th>Mean</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD¹</td>
<td>69.5 (3.68)</td>
<td>A</td>
<td>6</td>
<td>35.24 (1.01)</td>
<td>A</td>
<td>6</td>
</tr>
<tr>
<td>Broccoli</td>
<td>64.9 (5.42)</td>
<td>A</td>
<td>4</td>
<td>39.58 (9.20)</td>
<td>BC</td>
<td>4</td>
</tr>
<tr>
<td>Corn</td>
<td>50.9 (2.87)</td>
<td>B</td>
<td>5</td>
<td>25.80 (3.75)</td>
<td>C</td>
<td>5</td>
</tr>
<tr>
<td>Cotton</td>
<td>40.8 (3.81)</td>
<td>B</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Green beans</td>
<td>71.6 (6.10)</td>
<td>A</td>
<td>6</td>
<td>26.28 (6.21)</td>
<td>AB</td>
<td>6</td>
</tr>
<tr>
<td>Pigweed</td>
<td>16.1 (5.60)</td>
<td>C</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>

Means in a column followed by a common letter are not significantly different according to Fisher’s LSD (α=0.05).

¹Number of observations (N) for % nymphal survival and no. eggs/ female/ day = 6. An observation is the average for a colony.

¹ Artificial diet
DNA comparisons

The ML trees for \textit{cox1} and ND1 both displayed two clades of mtDNA sequences (data not shown). Clade 1 insects were restricted to the Delta, whereas clade 2 insects were found at all locations. The number of bases represented by the combined \textit{cox1} and ND1 regions is 1,045 which is about 6\% of the mitochondrial genome. One extract had a \textit{cox1} sequence from a parasitic hymenoptera (\textit{Euphorinae sp.}). The companion ND1 sequence was clearly \textit{L. lineolaris}. Figure 2.2 is a \textit{cox1} ML tree with samples of the \textit{L. lineolaris} clades along with other mirid species. The genetic distance between the clades is about 0.5\%. The genetic distance to other \textit{Lygus} species is 3 to 4\%. A haplotype network for the 72 \textit{cox1} sequences is presented in Figure 2.3. With one exception, clade 1 differs from clade 2 by three or more nucleotide substitutions. The ND1 clade separation was two nucleotide substitutions.
Figure 2.2    Maximum likelihood tree of Mississippi *L. lineolaris* and related mirids, mtDNA *cox1*.

The tree is condensed so that not all sequences are shown. The number of *L. lineolaris* sequences for each clade are indicated. Numbers at the nodes are the bootstrap (100) support percentages. HQ106003 *Nonlygus nubilus*, HQ105723 *Henrylygus nubilus*, HQ105900 *Lygocoris tinctus*. 
Figure 2.3  Haplotype network of mtDNA cox1 from Mississippi *L. lineolaris*.

Circle size is proportional to the number of individuals with that haplotype. The smallest circle is one insect. The number for the two largest circles is shown. Bars on the connecting lines indicate the number of nucleotide substitutions. Open area is Hills, shaded area is Delta.

**Discussion**

A primary objective of this research was to determine the biological effects of different diets on *L. lineolaris* populations collected from different regions across Mississippi. Diet had a significant effect on many of the variables measured, which was expected based on previous research (Khattat and Stewart 1977, Fleischer and Gaylor 1988, Abel et al. 2010, Adams et al 2014). However, differences in development to third instar was not expected because all treatments included artificial diet up to third instar. The more rapid development on broccoli suggests that nymphs obtained better nutrition from the broccoli than from the artificial diet. In contrast, nymphs fed green bean developed slower, suggesting that nymphs fed on the green beans, in spite of the beans apparently providing poorer nutrition than the artificial diet.
Region was not a significant factor for any variable measured. Unlike the data of Adams et al. (2014), these data indicated no biological differences between *L. lineolaris* populations. Adams et al. (2014) collected two colonies per region and each of the colonies in a region were collected from the “same general area”. In the present study three colonies from each region were compared and the sites of locations were at least 15 km from another collection site. Adams et al. (2014) showed that biological differences among diets in *L. lineolaris* populations exist, while the present study shows that, as a whole, *L. lineolaris* populations in the Delta region are not biologically different from those in the Hills region, with respect to developmental time, survival, fecundity, or hatch rate. The current experiments were not designed to compare colonies within a region, but there were apparent differences in performance among colonies within each region, so it is quite plausible that Adams et al. (2014) happened to find Delta colonies that performed different than Hills colonies while we could not attribute any differences to the region from where they were collected. The likelihood of encountering a population in Mississippi that is better adapted to cotton is still unknown, but based on the current study, they are not universally found throughout the Delta region.

The reason for the lack of oviposition of *L. lineolaris* reared on pigweed and cotton is unknown. Cotton squares were a moderately suitable diet based on nymphal developmental time and survival, while pigweed was a moderate to poorly suitable diet. Adams et al. (2014) successfully reared *L. lineolaris* on cotton squares, transferred adults to artificial diet, and had oviposition. In the present study adults were left on cotton squares and pigweed seed heads and no oviposition occurred. Females from these treatments were dissected and eggs were found to be present (unpublished data). A
possible reason for the lack of oviposition in the cotton and pigweed treatment could be due to the release of a plant volatile caused by the excision of the plant tissue. Herbivore attacks on plants have been shown to cause a release of plant volatiles that deter oviposition by pest insects (Kessler and Baldwin, 2001), excision of plant tissue has been shown to increase volatile response (Schmelz et al. 2001), and cotton has been shown to release volatile chemicals in response to herbivory (Rose et al. 1996). A second reason for the lack of oviposition may have been due to a lack of certain nutrients that may trigger oviposition. Female \textit{L. lineolaris} may have detected either high plant volatile levels or lack of good host nutrition which prevented oviposition in these treatments.

The \textit{cox1} region used in this study is the most commonly used barcode sequence for insects (Park et al. 2011). The \textit{cox1} primers are universal across insect species which accounts for the amplification of the \textit{Euphorinae} sequence from the parasitized \textit{L. lineolaris}. Because the ND1 primers were derived from the \textit{L. lineolaris} sequence, they only amplified the \textit{Lygus} DNA. The two mtDNA clades observed here correspond to the two clades reported on a national scope by Burange et al. (2012) who used a different part of the mt genome. Both clades are widely sympatric in North America. Clade 1 is more common in northern (colder) regions, whereas clade 2 is more common in southern (warmer) regions. Members of each clade have been collected from the same host plants at common sites which indicates that host preference is not a separating factor. Just as the Hills region collection sites in Mississippi contained only clade 2 individuals, a few other locations also appear to have single clades. All insects sampled from northern Alberta, Canada were clade 1 and all insects from Arizona were clade 2 (Richard R. Roehrdanz, USDA-ARS, Fargo, ND, unpublished data). The absence of one of the clades in some
places would appear to be random with no discernable cause. Compared to the genetic
distance between *L. lineolaris* and other *Lygus* species, the genetic distance between
clad 1 and clade 2 is small (Fig. 1). There is no evidence these two clades represent
isolated breeding populations. Taking all of these factors together, we do not believe that
the mtDNA data provide any evidence that Hills and Delta populations of *L. lineolaris*
are fundamentally distinct populations; however, it seems apparent that there is limited
gene flow between the two regions.

The lack of regional differences in biological and genetic comparisons is not
consistent with the observed disparity in economic losses to cotton between the Delta and
Hills populations of *L. lineolaris*. Insecticide resistance and changes in insect pest
management practices as a result of boll weevil eradication and the adoption of *Bt* cotton,
as well as changes in cotton acreage may be other reasons for the differences of the
impact on cotton of the two *L. lineolaris* populations. Decades of insecticide usage for
boll weevils and lepidopteran pests likely led to insecticide resistance development in *L.
lineolaris*, and boll weevil eradication (Snodgrass and Scott 2003) as well as changes in
IPM likely hastened additional insecticide resistance. Eradication of the boll weevil and
adoption of *Bt* cotton may have altered insect management practices for *L. lineolaris*
enough to allow populations of *L. lineolaris* to grow larger and become more problematic
in cotton. Also, cotton acreage has decreased in the state (Williams 1979-2012), thus the
density of *L. lineolaris* in cotton may be higher, but these factors do not fully explain the
differences in regional populations of *L. lineolaris* in Mississippi.

Landscape and land use in the two regions is not the same. The Delta is a region
consisting of 55% row crop agriculture, 2% forestry, 35% pasture/forage and other
herbaceous plants or fallow land, and 7% urban or otherwise unproductive land. The Hills is vastly different consisting of 20% row crop agriculture, 45% forestry, 30% pasture/forage and herbaceous plants or fallow land, and 5% urban or otherwise unproductive land (Fry et al. 2011). The difference in landscape ecology, when coupled with insecticide resistance, changes in control strategies, and natural processes, such as the impact of predators and parasitoids, may shed some light on the disparity between the regional impacts of *L. lineolaris* populations on cotton in Mississippi.

The larger proportion of agricultural land in the Delta may have led to a larger proportion of the *L. lineolaris* population in the Delta being exposed to insecticides than the Hills population. This may have increased the rate of insecticide resistance evolution for the Delta population. The larger proportion of non-row crop agricultural land (forest and pasture/other herbaceous) in the Hills may have provided more areas that sustained *L. lineolaris* not exposed to insecticides, thereby allowing larger proportions of the population to avoid insecticide resistance selection. The differences in landscape may also have other ecological effects on *L. lineolaris* populations. For example, beneficial insect populations and overall insect species have been shown to be affected by landscape factors (Ali and Reagan 1985, Fahrig and Jonsen 1998, Duelli et al. 1999, Menalled et al. 1999, Di Giulia et al. 2001, Carvalheiro et al. 2011). The Hills landscape may also provide a larger proportion of wild hosts in comparison to land used for cotton production than the Delta does. This would give a larger proportion of the *L. lineolaris* population in the Hills more opportunity to remain on wild hosts rather than move to cotton. The combination of landscape differences and insecticide usage may be the cause of the differences in cotton damage and control of the *L. lineolaris* populations.
In summary, differences could not be found biologically or genetically for regional populations of *L. lineolaris*. There were differences of developmental time, survival, and fecundity between the diets *L. lineolaris* was reared on. Clade 1 insects were not found in the Hills region, but both clades were found in the Delta, and at this time there is no explanation for the absence of clade 1 in the Hills. The differences that have been observed in the impact of *L. lineolaris* on cotton may be a result of increased insecticide resistance and changes in IPM resulting from the impact of the boll weevil eradication program and introduction of *Bt* cotton. Landscape differences between the two regions likely play a role in the level of insecticide resistance between the two regions as well as affect other aspects of *L. lineolaris* ecology.

**Acknowledgements**

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References


Abstract

*Lygus lineolaris* (tarnished plant bug) is the most serious insect pest of cotton (*Gossypium hirsutum* L.) in Mississippi but is a much more serious pest of cotton in the Delta region of the state. The reason for this is unknown but could be a result of a feeding adaption of the polygalacturonase (PG) system for cotton which occurs more predominantly in the Delta region than in the Hills region. Quantitative real-time PCR was carried out to measure the expression of PG genes in laboratory reared and field collected populations of *L. lineolaris*. An enzyme assay was also conducted to compare wild collected populations. Initial field and laboratory data revealed differences in region, sex, age, and host which guided the direction of our primary study during 2013 and 2014. Data revealed that the PG system is not adapted more for cotton in *L. lineolaris* in the Delta region than those in the Hills region. Analyses also revealed that the expression of PG genes was affected by the host the insects were collected from and that adults had higher PG expression than nymphs. Polygalacturonase enzyme activity was found not to be different between regions, hosts, or developmental stage.
Introduction

*Lygus lineolaris* (Palisot de Beauvois) (Hemiptera: Miridae), the tarnished plant bug, is a major pest of cotton (*Gossypium hirsutum* L.) in Mississippi. Mississippi cotton producers made an average of 4.3 insecticide applications for control of *L. lineolaris* each year from 2012 to 2014 at a cost of approximately $140 per ha per year (Williams 2012-2014). Cotton yield loss and cost of control of *L. lineolaris* is dissimilar between Mississippi’s two main cotton growing regions (Williams 1986-2014). Since 1986, the cotton insect losses for Mississippi have been reported separately for two regions, the Delta and the Hills. The Delta (Yazoo Basin) is the region of western Mississippi that is the flood plain of the Mississippi and Yazoo rivers and includes the area west of the Loess Bluffs (Figure 3.1). The Hills region, referred to in the cotton insect losses of Williams (1986-2014) beginning in 1986, is the remainder of the state (Figure 3.1), but, for the purposes of this study, primarily consists of the North Central Hills, Flatwoods, Pontotoc Ridge, Black Prairie, and Tombigbee Hills. The Delta is intensively farmed and has less non-cultivated areas than the Hills region, which typically has smaller fields with adjacent non-cultivated areas (Fry et al. 2011). The estimated number of insecticide applications targeting *L. lineolaris* has been higher in the Delta than in the Hills almost every year since 1986 (approximately 2.6-fold). (Williams 1986-2014). The reason for these differences is not yet clear.

Biological differences between populations of *L. lineolaris* collected in Mississippi have been reported (Adams et al. 2014). The number of days for *L. lineolaris* development to adulthood when feeding on cotton was 1.67 d longer for colonies collected from the Hills than for those collected from the Delta. Differences in
developmental time were not observed for those fed artificial diet. Additionally, females from colonies collected from the Delta laid 1.09 more eggs per day than females from colonies collected from the Hills. Shorter developmental time of Delta collected *L. lineolaris* fed cotton and higher fecundity in Delta colonies indicates that some populations of *L. lineolaris* may be more specialized for development on cotton and that the fecundity of some *L. lineolaris* populations are different. These biological differences between populations may indicate that some populations may be better adapted to some hosts than others. Another study has shown no significant biological differences between Delta and Hills colonies (Fleming et al. 2015).

A biological basis for possible population adaptations to hosts has not been identified. In phytophagous insects such as *Lygus* spp., which primarily feed directly on plant tissues using piercing sucking mouth parts, saliva plays a crucial role in extra-oral digestion of food. This mode of feeding has been described as “lacerate and flush”, whereby the flexible maxillae penetrates the plant tissues, destroying and breaking cell wall components and tissue by releasing large amount of saliva (Miles 1972). Thus, saliva helps allow the establishment of this pest on the host plant(s). The role of pectin degrading enzymes (polygalacturonases, PG) in the saliva of *Lygus* bugs was recognized more than four decades ago (Strong and Kruitwagen 1968; Strong, 1970). The direct effects of PGs produced during feeding can be square abscission, necrosis at the wound site, deformation of squares and bolls, aborted embryos, and reduced or altered vegetative growth (Strong 1970). Recent studies of the PG enzyme system have shown that the enzyme causes *Lygus*-like damage to plants (Shackel et al. 2005, Celorio-Mancera et al. 2008), that at least four PG genes (LIPG1, LIPG2, LIPG3, and LIPG4) exist in the saliva
of *L. lineolaris* (Allen 2007, Allen and Mertens 2008, Walker and Allen 2010), at least one of the genes (L1PG1) appears to be regulated based on diet (Walker and Allen 2010), and there are multiple isoforms of the *Lygus* PG enzyme and protein (Shackel et al. 2005, Celorio-Mancera et al. 2009). Little else is known about the functions of PG genes and the enzyme and how they are regulated. However, Walker and Allen (2010) found that PG genes may be regulated based on the plant species being fed upon. Thus, a hypothesized mechanism for the reported biological differences may be differences in the polygalacturonase enzyme system of *L. lineolaris* saliva. If PG is a mechanism driving the biological differences observed in *L. lineolaris* populations then investigations of this system may reveal which PG genes are most important for specific hosts, especially cotton, and if regional differences in the damage to cotton can be attributed to population differences in the PG system. Further knowledge of the PG system could support work toward understanding *L. lineolaris*-plant interactions as well as possibly help in developing novel RNAi based control strategies that can reduce or eliminate the ability of *L. lineolaris* to damage crops by inhibiting the genes needed to transcribe the PG enzyme. 

Previous research by Walker and Allen (2010) showed that PG1 expression levels could be reduced by approximately 80% with dsRNA. Further knowledge could also support the development of plant varieties, either through traditional plant breeding or genetic modification that can resist the effects of PG on plant tissue. Research has shown that polygalacturonase inhibiting proteins (PGIP) exist in plants and their inhibition can be specific to the source of the enzyme (Cervone et al. 1987, Toubart et al. 1992, Bergmann et al. 1994, Powell et al. 2000, D'Ovidio et al. 2004, Joubert et al. 2006, Ferrari et al. 2011, Akhgari et al. 2012). Frati et al. (2006) found that two PGIPs from green bean
(Phaseolus vulgaris L.) could inhibit mirid PG 10-42%; however two other PGIP they studied did not inhibit PG from mirids. Data (non-refereed) from the University of California-Davis indicates that PGIP in alfalfa (Medicago sativa L.), cotton, lima beans (Phaseolus lunatus L.), and black-eyed peas (Vigna unguiculata (L.) Walp.) are capable of inhibiting PG from L. hesperus, and that these traits are heritable, thus making it possible to selectively breed plants that are more tolerant to Lygus feeding (Teuber et al. 2002, Teuber et al. 2003, Teuber et al. 2004, Teuber et al. 2005, Teuber et al. 2007).

Additionally inhibition of PG by plants transgenically modified with PGIP has been shown effective. PGIP from grape (Vitis sp.), green bean and pear (Pyrus sp.) have been shown to inhibit fungal PGs in transgenically modified tobacco (Nicotiana tabacum L.), wheat (Triticum aestivum L.), tomato (Solanum lycopersicum L.), and canola (Brassica napus L.) (Powell et al. 2000, Joubert et al. 2006, Ferrari et al. 2011, Akhgari et al. 2012).

Based on the regional differences of the economic impact of L. lineolaris to cotton and our lack of knowledge of the PG system in L. lineolaris, a series of experiments were undertaken to look at changes in PG gene expression of L. lineolaris based on the regional location of the host population, plants from which the collection was made, insect age and sex. Differences in PG gene expression and enzyme activity between regional populations may indicate a potential biotype or sibling species shift and may help explain the higher severity of damage that occurs to cotton in the Mississippi Delta. A better understanding of the expression of PG genes in L. lineolaris from different host plants could reveal the key PG genes for that host and provide important insights in L. lineolaris-host plant interactions as well as provide insight for any future work with RNAi control strategies and development of PG resistant plants.
Figure 3.1  Map showing the geographic regions of Mississippi.

The Yazoo basin is the area referred to as the Delta. The region referred to as the Hills is the rest of the state, but concerning cotton production is primarily the Flatwoods, Pontotoc Ridge, and Black Prairie geographic regions, as these are the primary cotton production areas in the Hills region. Source: Hutson (2008).
Materials and methods

2012 L. lineolaris field collections

Lygus lineolaris adults were collected from 30 locations (five locations per host per region) in the Delta and Hills regions of Mississippi from cotton (Gossypium hirsutum L.), horseweed (Conyza canadensis (L.) Cronquist), and pigweed (Amaranthus sp.) in the summer of 2012. Collections were made with a 38 cm sweep net, insects were placed into plastic tubs to be separated from other insects and sweeping trash and then aspirated into cricket cages. Reproductive plant tissue from the host plants was placed in the cages and the cages were transported back to the laboratory in a cooler containing ice packs. Cages were placed in a -20° C freezer overnight to kill the insects. The following day, ten insects from each host and location were placed into scintillation vials and stored in a -80° C freezer until further processing.

Laboratory assays

Sex and age comparisons

The first experiment compared sex and age of L. lineolaris to determine factors that could help explain the variation in field collected data. Lygus lineolaris adults from a long term colony housed at the Mississippi State University Insect Rearing Center were used to compare expression of PG genes in male and female L. lineolaris adults at four different ages. The colony is housed in a rearing facility with a 16:8 light:dark cycle that is kept at 26.7° C with approximately 60-70 % relative humidity. Insects were fed an oligidic artificial diet (Cohen 2000) and offered carrageenan gel packs for oviposition. Insects were aspirated from containers of the parent colony containing approximately one-day-old, five-day-old, ten-day-old, and fifteen-day-old adults. Ten of each sex at
each age were removed for each sample, and this was repeated on each of three days (replicates). Insects that were removed from the parent colonies were placed in scintillation vials in a -20° C freezer to kill them and the following day they were transferred to 1.5 ml DNase and RNase micro-centrifuge tubes and placed in a -80° C freezer for storage until further processing.

**Nymph and adult comparisons**

The second experiment to evaluate PG gene expression compared adult and nymphal *L. lineolaris* from two colonies that had been collected in the Spring of 2012. These colonies were housed for approximately four generations at the Mississippi State University Insect Rearing Center under the same conditions as the previously mentioned colony. Insects were aspirated from each of three cohorts (replicates) per colony from containers of old adult (>10 days) young adult (<five days) and nymph (5th instar) containers within each colony and placed into scintillation vials in a -20° C freezer overnight to kill them. The following day the insects were placed into 1.5 ml DNase and RNase free micro-centrifuge tubes for storage in a -80° C freezer until further processing.

**2013-2014 field collections**

To further explore data that was collected in initial experiments, a two year study to evaluate PG gene expression and enzyme activity in sexed adult and nymphal *L. lineolaris* collected in the wild from hosts was conducted. Adults and fifth instar nymphs of *L. lineolaris* were collected from crimson clover (*Trifolium incarnatum* L.), cotton, fleabane (*Erigeron* sp.), horseweed, and pigweed in the late Spring, Summer, and early Fall of 2013 and 2014. Insects were collected, transported, and killed similar to those in
the 2012 study. The day following collection, dead insects were sorted by sex/developmental stage (male, female, 5th instar nymph) and ten or twenty of each sex/developmental stage from each host and location were placed into scintillation vials. Males and females were separated by observing the presence or absence of the ovipositor on the ventral surface of the abdomen (Schuh and Slater 1995). Vials were then placed into a -80° C freezer until further processing for gene expression or enzyme assay.

**Gene expression**

*Dissection and isolation of salivary glands*

Insects in all experiments were surface sterilized by dipping each insect into 70% ethyl alcohol and then rinsed by dipping each insect into distilled water. Insects were then air dried under a laminar flow hood and sorted to be used either to measure gene expression or enzyme activity. Salivary glands were extracted by removing the head and prothorax by pinching the projection of the prothoracic notum and pulling forward. Ten sets of head and thorax tissue containing salivary glands for each sample were placed in 1.5 ml DNase and RNase free micro-centrifuge vials, and then placed in a -80° C freezer until further processing for gene expression. For the 2013-2014 wild *L. lineolaris* study, another ten salivary glands for each sample were placed in micro-centrifuge tubes and stored in a -20° C freezer until further processing for enzyme activity.

*RNA isolation*

All insects from all experiments, with the exception of those from the 2013-2014 experiment that were used to assay enzyme activity, were homogenized in 500 µl of Tri Reagent (Trizol) (Sigma Aldrich, St. Louis, MO) with a Kimble Kontes cordless
motorized pestle with polypropylene pestle tips (Sigma-Aldrich, St. Louis, MO). Samples stood at room temperature for five minutes and 100 µl of molecular biology grade chloroform (Fisher Scientific, Waltham, MA) was added. Samples were then shaken for 15 sec, allowed to stand at room temperature for three minutes, and then centrifuged at 12,000 RPM for 15 min at 4°C. Approximately 200 µl of the top layer of aqueous solution was then pipetted into a new 1.5 ml DNase and RNase free micro-centrifuge tube and 200 µl of biotechnology grade isopropanol (Amresco, Solon, OH) was added to the new tube. The isopropanol solution was mixed, stood at room temperature for 20 min, and then centrifuged at 14,000 RPM for 15 min at 4°C. The supernatant was then decanted and remaining pellet washed twice in 75% ethanol using a microfuge. Ethanol was decanted and the remaining ethanol pipetted out if necessary. Pellets were allowed to dry for 15-20 minutes under a laminar flow hood to remove any remaining ethanol. After drying, 150 µl of nuclease free water (Qiagen, Germantown, MD) was added to the tubes which then stood at room temperature for 15 minutes. Tubes were vortexed until the pellet dissolved and were then spun down in a microfuge. Tubes were either immediately moved to the genomic DNA removal step or stored in a -80°C freezer until they could be processed further.

Removal of genomic DNA

Genomic DNA was removed using a Thermoscientific procedure and kit (Thermo Fisher Scientific, Waltham, MA). 80 µl of RNA was placed in 1.5 ml DNase and RNase free micro-centrifuge tubes with 10 µl of 10x MgCl₂ master mix buffer, 4 µl of DNase I, and 6 µl of nuclease free water. This solution was then vortexed and microfuged. 50 µl of solution was transferred to 200 µl DNase and RNase free PCR tubes (Bio-Rad, Hercules,
CA) and tubes were placed on a thermocycler for 30 min at 37° C. 10 µl of 50mM EDTA (supplied in kit) was added to each PCR tube and tubes were incubated at 65 °C for 10 min. The 50 µl of RNA was transferred into 1.5 µl RNase and DNAse free micro-centrifuge tubes and 125 µl of 100% ethanol and 5µl of 3M sodium acetate (Sigma Aldrich, Waltham, MA) were added to the tubes. The solution was mixed in a microfuge and placed in a -20° C freezer for at least 2 hr or overnight. Tubes were then centrifuged at 19,300 g [rcf] for 30 min at 4° C. The supernatant was decanted and the remaining pellet washed twice with 75% ethanol in a microfuge. Excess ethanol was pipetted out and the pellet was allowed to air dry 15 min under a laminar flow hood to ensure all ethanol was removed. 80 µl of nuclease free water was added to the tubes and tubes were allowed to stand for 15 min at room temperature to soften the pellet. Tubes were then vortexed until the pellet dissolved and were spun down in a microfuge and stored in a -80° C freezer until further processing.

_cDNA synthesis_

To perform cDNA synthesis the quantity of RNA was first estimated using Take-3 (Biotek Synergy H1 Hybrid Reader, Winooski, VT). RNA was transferred to RNAse and DNase free 1.5 ml micro-centrifuge tubes and equalized by diluting with nuclease free water. iScript cDNA synthesis was performed using a kit and procedure from Bio-rad (Hercules, CA). Four µl of 5x reaction mix, 6 µl of nuclease free water, and 1 µl of iScript reverse transcriptase and 10 µl of RNA were added to 200 µl PCR tubes and the tubes were placed on a thermocycler. Tubes were incubated at 25° C for 5 min, 42° C for 30 min, and 85° C for 5 min, and then cooled to 4° C. cDNA was diluted to 5 ng per µl
by diluting with nuclease free water in DNase and RNase free 1.5 µl micro-centrifuge tubes.

**qRT-PCR**

Measurement of PG gene expression was performed using qRT-PCR with an Eppendorf Realplex² Mastercycler (Eppendorf, Hamburg, Germany). A master mix for each PG gene was prepared by mixing 7.5 µl of SYBR Green (Thermo Fisher Scientific, Waltham, MA), 4.9 µl of nuclease free water, and 0.6 µl of the appropriate primer (IDTDNA, www.idtdna.com) for each well of Eppendorf 96-well twin-tec skirted plates (Eppendorf, Hamburg, Germany). After the master mix was pipetted, 2 µl of cDNA was pipetted into each well. A film (supplied with the plates) was placed over the plate, the plate was then agitated by vortexing, centrifuged for 5 min at 3000 rpm, and placed into the Eppendorf Realplex² Mastercycler. The qRT-PCR program was then set to run at 95° C for 10 min then perform 45 cycles at 95° C for 15 sec, 59° C for 20 sec, and 72° C for 30 sec, and then measure a melting curve by running at 95° C for 15 sec then 60° C for 15 sec, and then ramped for 20 min up to 95° C and hold for 15 sec. Output of the qRT-PCR was corrected for drift, placed on log scale, and the threshold was set at 500 nm. Gene expression was measured using the relative quantification method of Livak and Schmittgen (2001).

**Enzyme activity**

Activity of PG enzyme was measured based on the methods of Vinokurov et al. (2014). Ten insect salivary glands (in microcentrifuge tubes) per sample were homogenized in 200 µl of 50 mM phosphate buffer using a motorized pellet pestle. Tubes
were centrifuged at 600 g [rcf] for 10 minutes. A series of D-galacturonic acid (Sigma Aldrich, St. Louis, MO) standards was created by diluting 60 μl of 0.1 % D-galacturonic solution in 240 μl of 50 mM sodium acetate buffer (highest concentration). Six additional concentrations were made, each a 50 % concentration of the previous. The lowest concentration was 100 % sodium acetate buffer. 120 μl of 0.5 % polygalacturonic acid solution (Sigma Aldrich, St. Louis, MO) was added to 600 μl tubes of both samples and standards, and tubes were equilibrated to 30° C for ten minutes. 30 μl of sample supernatant was added to sample tubes and 30 μl of 50 mM sodium acetate with 0.05 % bovine serum albumin (Thermo Fisher Scientific, Waltham, MA) (pH 5.0) was added to standard tubes. All tubes were vortexed and incubated for 10 min at 30° C. 150 μl of a 16 mM copper sulfate, 1300 mM sodium sulfate, 226 mM sodium carbonate, 190 mM sodium bicarbonate, 43 mM sodium potassium tartarate solution was added to all tubes. The tubes were then heated at 95° C for 15 min and allowed to cool for approximately 15 minutes. 150 μl of a 40 mM molybdic acid, 19 mM Arsenic acid, 756 mM sulfuric acid solution was added to all tubes. Tubes were mixed by inversion three times and lids were opened to release the pressure that formed from the reaction. Tubes were then vortexed and centrifuged at 6000 g [rcf] for 5 min. 100 μl of substrate was pipetted in triplicate into wells of a 96-well Fisher brand clear, flat-bottom plate (Thermo Fisher Scientific, Waltham, MA). Absorbance was read at 540 nm in a BioTek Synergy H1 multi-mode plate reader (Biotek, Winooski, VT). Protein was estimated using the BCA method (Smith et al. 1985). Absorbance was calculated by calculating a standard curve and dividing the micromoles of D-galacturonic acid released by the dilution factor of 0.1. PG activity is expressed as units of D-galacturonic acid per mg of protein.
**Statistical analyses**

Statistical analyses of all experiments were performed in SAS 9.3 using the Proc Glimmix procedure (SAS Institute Inc., Cary, NC). Data for all gene expression experiments were log transformed to meet the assumptions of equal variance. The Kenward-Roger method was used to calculate degrees of freedom (Kenward and Roger 1997). The normalizer used for all experiments was a ΔCt (difference in the number of cycle threshold between the gene of interest and the housekeeping gene) value from a previously studied colony reared on artificial diet. Data points that failed to amplify or exhibited irregular amplification (standard deviation>0.5) among the technical replicates of each sample were eliminated prior to data analysis, as per instructions of the program manufacturer (available from www.lifetechnologies.com).

For the 2012 wild *L. lineolaris* study, replicate (location within a region) was used as a random factor. Region (Delta and Hills), host (cotton, horseweed, and pigweed), and gene (PG1, PG2, and PG3) were analyzed for main effects and interactions. Data for each gene were analyzed for normality of distribution and one data point was removed because it was more than three standard deviations from the predicted value.

For the laboratory colony, comparisons of sex and age, the interactions of sex, PG gene, and age were analyzed for interactions and main effects. Replicate was used as a random factor. For the adult and nymphal comparisons, colony and replicate were used as random factors and replicate, colony, and replicate(colony) were the terms used in the random statement. Developmental stage of the insects and PG genes were analyzed for interactions and main effects.
There was insufficient data to evaluate a year effect in the 2013-2014 wild collected experiment, thus replicate, year, and replicate(year) were initially used as the random factors. Clover was only present for samples from the Hills region, thus clover was removed from the analyses. For initial analyses of gene expression, region (Delta and Hills), host (cotton, fleabane, horseweed, and pigweed), type (male, female, and nymph), and gene (PG1, PG2, PG3) were analyzed for interaction and effects. Region as a main effect and in interactions was never significant (Table 3.1), therefore region was removed from the fixed effects statement and the factors region and region*rep(year) were added to the random statement. Since region was no longer a factor being examined, data for clover was placed back in the dataset and host, type, and gene were analyzed for effects and interaction. Data were analyzed for normality of distribution and two data points were removed because they occurred outside of three standard deviations.

Polygalacturonase enzyme activity in the 2013-2014 wild collected study was also compared. Again, there was insufficient data to make any comparisons between years, thus initially replicate, year, and replicate(year) were used as random factors. Again, clover was removed from the analysis since it was found only in the Hills region. Initial analyses revealed no effect of region (Table 3.1), therefore region was removed from the fixed effects statement, clover was placed back into the analysis, and region and region*rep(year) were added to the random statement. Host and type were analyzed for interactions and main effects.
Results

2012 field collected *L. lineolaris* gene expression

The interaction of region and host had a significant effect (Table 3.1) on overall gene expression. The average overall PG expression for *L. lineolaris* collected from cotton was significantly higher for those collected from the Hills (Figure 3.2), and the average overall PG expression in *L. lineolaris* collected from the Delta was significantly higher for those collected from horseweed than from cotton (Figure 3.2). Overall, expression of PG1 was significantly higher (Table 3.1) than expression of PG2 and PG3 (Figure 3.3). These data indicate there are differences between individual genes, as well as regional and host differences in average overall gene expression.

Table 3.1  F-statistics for 2012 wild *L. lineolaris* gene expression.

<table>
<thead>
<tr>
<th></th>
<th>df</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>region<em>host</em>gene</td>
<td>4, 61.93</td>
<td>1.26</td>
<td>0.30</td>
</tr>
<tr>
<td>host*gene</td>
<td>4, 65.83</td>
<td>0.75</td>
<td>0.56</td>
</tr>
<tr>
<td>region*gene</td>
<td>2, 65.64</td>
<td>2.76</td>
<td>0.07</td>
</tr>
<tr>
<td>region*host</td>
<td>2, 72.20</td>
<td>5.36</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>host</td>
<td>2, 71.77</td>
<td>1.26</td>
<td>0.29</td>
</tr>
<tr>
<td>gene</td>
<td>2, 71.86</td>
<td>13.41</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>
Figure 3.2  
Back-transformed mean and standard error of the effects of host and region on total relative PG gene expression in wild *L. lineolaris* in 2012.

Columns with the same letter are not significantly different (Fisher’s LSD, α=0.05). N=14 for Delta cotton and horseweed, N=13 for Delta pigweed and Hills cotton, and N=15 for Hills horseweed and pigweed.
Columns with the same letter are not significantly different (Fisher’s LSD, α=0.05). N=30 for PG1, N=28 for PG2, and N=26 for PG3.

**Sex and age gene expression comparisons**

The interaction of sex and age had a significant effect on the average overall gene expression (Table 3.2). The sex and age interaction revealed differences in average overall PG expression between sexes at individual ages as well as differences within a sex at different ages (Figure 3.4). Average overall expression of PG was higher in one-day-old females than in one-day-old males; however, expression was higher in five-day-old males than five-day-old females. Average overall expression significantly decreased between one-day-old and five-days-old females but remained steady thereafter. Average overall expression significantly increased in males between one-day-old and five-day-old, but returned to a level similar to that in one-day-old males. The expression of individual genes was significantly different (Table 3.2) with expression of PG1 higher than expression of PG2 or PG3 (Figure 3.5). Data indicate that average overall PG expression
is different between males and females at specific points in time. F-statistics for sex and age comparisons of *L. lineolaris* gene expression.

Table 3.2  F-statistics for sex and age comparisons of *L. lineolaris* gene expression.

<table>
<thead>
<tr>
<th>Sex and age gene expression comparisons</th>
<th>df</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>sex<em>gene</em>age</td>
<td>6, 46</td>
<td>0.34</td>
<td>0.91</td>
</tr>
<tr>
<td>gene*age</td>
<td>6, 52</td>
<td>0.32</td>
<td>0.92</td>
</tr>
<tr>
<td>sex*gene</td>
<td>2, 52</td>
<td>0.47</td>
<td>0.63</td>
</tr>
<tr>
<td>sex*age</td>
<td>3, 52</td>
<td>10.3</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>sex</td>
<td>1, 52</td>
<td>0.04</td>
<td>0.84</td>
</tr>
<tr>
<td>age</td>
<td>3, 52</td>
<td>2.22</td>
<td>0.10</td>
</tr>
<tr>
<td>gene</td>
<td>2, 52</td>
<td>16.82</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Figure 3.4  Back-transformed mean and standard error of the total relative PG gene expression of reared *L. lineolaris* from the study of age and sex.

Columns followed by the same letter are not significantly different (Fisher’s LSD, α=0.05). N=9 for all treatments.
Figure 3.5  Back-transformed mean and standard error of the expression of PG genes in reared *L. lineolaris* in the study of age and sex.

Columns with the same letter are not significantly different (Fisher’s LSD, α=0.05). N=24 for each gene.

**Nymph and adult gene expression comparisons**

The average overall expression of PG was not significantly different between adults of either age and nymphs (Table 3.3). The expression of individual genes was significantly different (Table 3.3) with the expression of PG1 significantly higher than expression of PG2 and PG3 (Figure 3.6). These data indicate significant differences between expression of individual genes but do not reveal any differences in average overall gene expression at different ages/developmental stages.

Table 3.3  F-statistics for nymph and adult comparisons of *L. lineolaris* gene expression.

<table>
<thead>
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<th>Nymph and adult gene expression comparisons</th>
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<th>p</th>
</tr>
</thead>
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<tr>
<td>stage*gene</td>
<td>4, 38.99</td>
<td>0.76</td>
<td>0.56</td>
</tr>
<tr>
<td>stage</td>
<td>2, 43.05</td>
<td>0.70</td>
<td>0.50</td>
</tr>
<tr>
<td>gene</td>
<td>2, 42.99</td>
<td>20.56</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>
Figure 3.6  Back-transformed mean and standard error of the expression of PG genes in *L. lineolaris* in the study of nymphs and adults.

Columns with the same letter are not significantly different (Fisher’s LSD, α=0.05). N=17 for PG1 and PG2 and N=16 for PG3.

**2013-2014 wild collected *L. lineolaris* gene expression and enzyme activity**

The interaction of host and gene and host and type had a significant effect on PG expression (Table 3.4). There was not a gene that appeared to be the most important gene for *L. lineolaris* collected from cotton; however, the relative expression of individual genes was significantly different within all the other hosts (Figure 3.7). Generally, the average overall expression of PG was significantly higher for males and females than for nymphs but expression between males and females was never different (Figure 3.8). There were no significant effects or interactions in regards to activity of PG enzyme (Table 3.4). The units of D-galacturonic acid per mg of protein ranged from 0.33 to 0.45.
These data indicate that there are individual genes that are more important for some host plants than others, but overall PG enzyme activity is similar over all hosts.

Table 3.4  F-statistics for 2013-2014 wild *L. lineolaris* gene expression and enzyme activity.

<table>
<thead>
<tr>
<th></th>
<th>df</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>2013-2014 gene expression from wild <em>L. lineolaris</em></strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Effects of region</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>region<em>host</em>type*gene</td>
<td>12, 380.6</td>
<td>0.35</td>
<td>0.98</td>
</tr>
<tr>
<td>region<em>host</em>gene</td>
<td>6, 393.9</td>
<td>0.31</td>
<td>0.93</td>
</tr>
<tr>
<td>region<em>host</em>type</td>
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<td>1.35</td>
<td>0.23</td>
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<td>4, 380.7</td>
<td>0.60</td>
<td>0.66</td>
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<td>region*type</td>
<td>2, 438.5</td>
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<td>0.19</td>
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<td>3, 438</td>
<td>0.37</td>
<td>0.78</td>
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<tr>
<td>region*gene</td>
<td>2, 426.9</td>
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<tr>
<td>region</td>
<td>1, 456</td>
<td>0.03</td>
<td>0.90</td>
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<tr>
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<td>16, 484.9</td>
<td>0.41</td>
<td>0.98</td>
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<tr>
<td>type*gene</td>
<td>4, 500.7</td>
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<tr>
<td>host*gene</td>
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<td>host*type</td>
<td>8, 516.8</td>
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<td>0.04</td>
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<td><strong>2013-2014 enzyme activity from wild <em>L. lineolaris</em></strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Effects of region</td>
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<td>5, 107.1</td>
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<td>0.74</td>
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<td>region*type</td>
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<td>0.37</td>
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<td>region*host</td>
<td>3, 115.6</td>
<td>0.85</td>
<td>0.47</td>
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<tr>
<td>region</td>
<td>1, 126.6</td>
<td>0.19</td>
<td>0.66</td>
</tr>
<tr>
<td>Effects with region in random statement</td>
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<td></td>
</tr>
<tr>
<td>host*type</td>
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<td>0.75</td>
<td>0.63</td>
</tr>
<tr>
<td>type</td>
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<td>0.06</td>
<td>0.94</td>
</tr>
<tr>
<td>host</td>
<td>4, 127.5</td>
<td>1.88</td>
<td>0.11</td>
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</tbody>
</table>

Discussion

Laboratory data revealed differences in PG expression between sexes, with age and developmental stage, and between individual PG genes. These results were anticipated based on data from Allen and Mertens (2008) and Walker and Allen (2010). Differences of age and sex may help explain some of the variation found in the studies of wild collected *L. lineolaris*. It is not possible to know the age of wild collected insects, thus the laboratory data help explain the variation in wild collected *L. lineolaris* that cannot be accounted for in wild collected insects. However, sex can be accounted for,
thus sex was included as a factor in the 2013-2014 wild collected study. Comparisons of PG expression in adults and nymphs were found not to be significantly different in our study, but we assumed that the possibility existed for more stable PG expression in nymphs than adults in wild populations because nymphs predominantly feed on one host while adults are capable of changing hosts, thus data from wild collected nymphs may have less variation than adults. For this reason nymphs were also collected for the 2013-2014 wild collected study. Data from our laboratory colony experiments revealed that consideration must be given to age and sex when planning and implementing PG experiments.

Figure 3.7 Back-transformed mean and standard error of the effects of host on relative PG gene expression in wild collected L. lineolaris from Mississippi during 2013-2014.
Columns with the same letter are not significantly different (Fisher’s LSD, α=0.05). N ranges from 27 to 58.

Figure 3.8  Back-transformed mean and standard error of the effects of host on type on relative total PG gene expression in wild *L. lineolaris* collected from Mississippi during 2013-2014.

Columns with the same letter are not significantly different (Fisher’s LSD, α=0.05). N ranges from 14 to 68.

Table 3.5  Mean and standard error (SE) of the enzyme activity of PG in wild *L. lineolaris* collected in Mississippi during 2013-2014.

<table>
<thead>
<tr>
<th>Host</th>
<th>N</th>
<th>Mean (±SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clover</td>
<td>19</td>
<td>0.42 (0.02)</td>
</tr>
<tr>
<td>Cotton</td>
<td>15</td>
<td>0.35 (0.03)</td>
</tr>
<tr>
<td>Fleabane</td>
<td>45</td>
<td>0.38 (0.01)</td>
</tr>
<tr>
<td>Horseweed</td>
<td>29</td>
<td>0.34 (0.02)</td>
</tr>
<tr>
<td>Pigweed</td>
<td>30</td>
<td>0.35 (0.02)</td>
</tr>
</tbody>
</table>

Expressed in units of D-galacturonic acid released per mg of protein.
Data from the two experiments using wild collected *L. lineolaris* revealed differences in average overall PG expression between hosts (2012 data), differences in individual gene expression within hosts (2012 and 2013-2014 data), and differences in average overall gene expression between males and females versus nymphs within hosts (2013-2014 data). These differences were expected based on laboratory data as well as data from Allen and Mertens (2008) and Walker and Allen (2010). Data from 2012 indicated that PG1 was expressed at the highest rates of all three genes in *L. lineolaris* collected from cotton, but this was not true for the 2013-2014 data. This incongruence fails to reveal a PG gene that may be the most important in *L. lineolaris* feeding on cotton and that could be effectively targeted with a RNAi insecticide or a PGIP variety of cotton. A RNAi insecticide or PGIP variety of cotton that targets multiple genes or the PG system as a whole would likely be necessary to effectively manage *L. lineolaris*.

Wild collected males and females did not express PG differently but had higher overall PG expression than nymphs collected from all hosts except pigweed. One explanation for this may be that adults need higher levels of PG genes ready to be translated to protein in the event that a host change is necessary. Nymphs would not normally be able to change host plants. The lower expression of PG in nymphs could make nymphs a more suitable target than adults for RNAi insecticides or PGIP cotton. The lower expression of PG genes in nymphs could be easier to inhibit and could require lower doses of RNAi insecticide or lower expression of PGIP in the transgenic plants.

The lack of significant differences in enzyme activity reveals that the concentration of PG enzyme in *L. lineolaris* saliva is not dependent upon host. This indicates, in light of the gene expression data, the amount of enzyme is limited within the
saliva but the makeup of the enzyme may vary based on expression of the specific genes for the host. Our enzyme assay is only able to look at the total amount of PG enzyme through the measurement of D-galacturonic acid. Some research has indicated that there are multiple isoenzymes or proteins of PG in some PG expressing organisms, including *L. lineolaris* (Agblor et al. 1994, De Lorenzo et al. 2001, Poinssot et al. 2003, Shackel et al. 2005, Celorio-Mancera et al. 2009). Based on this information there may be a limit to the total concentration of PG enzyme in the saliva of *L. lineolaris* and that each PG gene translates a specific isoenzyme for use on a specific host, thus the expression of PG genes and the concentrations of isoenzymes would vary on hosts, whereas total concentration of PG enzyme would not vary. More research in this area could be useful for discovering the functions of PG genes and their possible isoenzymes, as well as developing RNAi techniques or *L. lineolaris* resistant PGIP varieties of cotton.

In summary, expression of PG genes can be affected by host, developmental stage, age, and sex. These potential sources of variation need to be considered before implementing PG experiments. Developmental stage, age, and sex should be used as blocks in experiments when appropriate. Region did not have a consistently significant effect on PG gene expression, thus the PG system does not appear to be an important factor in the adaptation of a suspected cotton biotype or sibling species shift toward host preference of cotton in the Mississippi Delta region. Host has a strong effect on expression of all three PG genes evaluated in this study; however, none of the genes stood out as the most important to *L. lineolaris* feeding on cotton. More research is needed to understand the PG system and the potential to target the system with RNAi insecticides or PG resistant PGIP plants. Future PG research should include evaluating
cotton varieties for levels of PGIPs and PG resistance as well as identification of PGIPs in cotton that may be useful in developing genetically modified cotton that can resist PG damage. Also work with RNAi comparing the effects of targeting single versus multiple genes of PG should be conducted.

**Acknowledgements**

Thanks to Kathy Knighten and the many student workers who maintained the rearing facilities and colonies. Thanks to Elizabeth Meadows, Sara Ford, Emily Ousterhout, and Chloe Henson for technical assistance in preparing samples for analysis. Partial funding for this research was provided by USDA-ARS Specific Cooperative Agreement 58-6402-3-017.
References


CHAPTER IV
COMPARISONS OF INSECTICIDE SUSCEPTIBILITY AND DETOXIFICATION ENZYMES IN MISSISSIPPI POPULATIONS OF LYGUS LINEOLARIS

Abstract

Lygus lineolaris (Palisot de Beauvois) is a serious pest of cotton (Gossypium hirsutum L.) in Mississippi but is considered to be more difficult to manage in the Delta region of the state. This may be due to a faster rate of insecticide resistance evolution in L. lineolaris populations in the Delta region. During 2014, experiments were undertaken to compare LC50s of L. lineolaris populations to acephate, permethrin, and imidacloprid, as well as the esterase and glutathione s-transferase (GST) activity of these populations. The results of these studies indicated that LC50s were not different between regions for any of the insecticides. The results of the detoxifying enzyme activity assays revealed that there is a strong trend for higher GST activity in Delta populations of L. lineolaris, while assays for esterase activity revealed that esterase activity was significantly higher in Delta populations. These results indicated that LC50s may not be as sensitive of a method for making comparisons of insecticide tolerance as the use of biochemical assays for detoxifying enzymes.
Introduction

*Lygus lineolaris* (Palisot de Beauvois) (Hemiptera: Miridae) has become a major pest of cotton (*Gossypium hirsutum* L.) in Mississippi. Since 1979 it has frequently been considered one of the three most costly insect pests of Mississippi cotton (Williams 1997, Williams 1986-2014). The estimated losses and costs of control from *L. lineolaris* damage are not uniform across the state. Since 1986 the cotton insect losses of Mississippi have been separated into two regions, the Delta and the Hills. The Delta is the region of western Mississippi that is the flood plain of the Mississippi and Yazoo rivers and includes the area west of the Loess Bluffs (Figure 4.1). The Hills region, referred to in the cotton insect losses of Williams (1986-2014) beginning in 1986, is the remainder of the state. The Delta region is intensively farmed and has less non-cultivated areas than the Hills region, which typically has smaller fields with adjacent non-cultivated areas. The estimated number of insecticide applications targeting *L. lineolaris* in cotton in the Delta region is estimated to be 2.6-fold higher than in the Hills region over the last 29 years (Williams 1986-2014). Insecticide use in the Delta has increased at a rate of approximately 0.3 applications per year since 1999, while in the Hills usage has increased by approximately 0.2 applications per year since 2002 (Figure 4.2). Prior to this insecticide usage for *L. lineolaris* were increasing by approximately 0.1 applications per year in the Delta and decreasing by approximately 0.05 applications per year in the Hills (Figure 4.2).

The reason for the higher pest pressure in the Delta region and increasing pest pressure in both regions is largely unknown. Higher insecticide tolerance and the presence of resistant populations in the Delta are often considered a cause for the
dissimilarity between the regions. Insecticide resistance in *L. lineolaris* in the United States, especially in the Mississippi Delta region, has been confirmed for several classes of insecticides, including: pyrethroids, organophosphates, organochlorines, carbamates, and cyclodienes (Cleveland and Furr 1980, Cleveland 1985, Snodgrass and Scott 1988, Snodgrass 1994, Snodgrass and Elzen 1995, Dennehy and Russell 1996, Pankey et al. 1996, Snodgrass 1996a, b, Hollingsworth et al. 1997, Holloway et al. 1998, Snodgrass and Scott 1999, 2000, 2002, 2003, Snodgrass et al. 2009). Only one study comparing insecticide resistance in Delta and Hills populations is known to have been published. Snodgrass et al. (2009) compared populations of *L. lineolaris* from both regions from 2005 to 2007. The populations from the Hills were primarily from the northern portions of the Loess Bluff and North Central Plateau geographical regions (Figure 4.1). They found that in 2005 the average LC$_{50}$ for acephate in the Hills population was higher than in the Delta population. Also in 2005, both regions had some populations with resistance ratios for acephate of >3.0, which, according to Snodgrass et al. (2009), may not be manageable with labeled rates of insecticides. However, in 2006 and 2007, the average LC$_{50}$s and resistance ratios were higher in Delta populations. They also found that the percent mortality in *L. lineolaris* treated with permethrin was lower in Delta collected populations. These data indicate that LC$_{50}$s, resistance ratios, and mortality vary in both Delta and Hills populations of *L. lineolaris*, but, overall, *L. lineolaris* from the Delta tend to be more tolerant of insecticides.

Other research also shows variation in mortality, resistance ratios, and LC$_{50}$s of *L. lineolaris* populations. Zhu and Luttrell (2015) showed mortality for two imidacloprid resistant Delta populations to be 6 and 62 %, and both had significantly lower mortality
than the susceptible colony. Zhu and Luttrell (2012) found that some Delta populations had more susceptibility to acephate than the susceptible laboratory colony while other populations had resistance ratios ranging from 1.6- to 5.9-fold higher than the laboratory colony. Snodgrass (1994) found resistance ratios of 54 for permethrin and 35 for bifenthrin, both pyrethroids, between two colonies of *L. lineolaris* collected from the Delta. Snodgrass (1996a) found significantly different levels of insecticide resistance in populations of *L. lineolaris* from four different Delta cotton fields. Snodgrass and Scott (2002) found resistance ratios ranging from 0.7 to 3.1 in fourteen populations of *L. lineolaris* from the Delta region. Data from Hills populations of *L. lineolaris* indicates that LC₅₀s vary from 1.7 to 23.5 µg/vial for acephate, 0.8 to 10.0 µg/vial for permethrin, and 0.03 to 3.82 µg/vial for imidacloprid (Fred R. Musser, Mississippi State University, unpublished data). These data taken together show that levels of insecticide tolerance are highly variable within regional populations.

If insecticide resistance is a factor in population differences in *L. lineolaris*, then levels of insecticide detoxification enzymes may also be different. Three enzyme groups are associated with metabolic insecticide detoxification: cytochrome P-450s (Feyereisen et al. 1995, Tomita et al. 1995, Feyereisen 1999, Kasai and Scott 2000, Puinean et al. 2010a), esterases (Campbell et al. 1998, Raghavendra et al. 1998, Valles 1998, Smyth et al. 2000, Karunaratne and Hemingway 2001, Liu and Han 2003), and glutathione s-transferases (Oppenoorth et al. 1972, Fournier et al. 1987, Armstrong 1997). Gene expression, mRNA levels, and activity of these enzymes have all been shown to be higher in resistant *L. lineolaris* strains and with exposure to insecticides (Zhu and Snodgrass 2003, Zhu et al. 2004, Zhu et al. 2007, Zhu et al. 2011, Zhu et al. 2012, Zhu and Luttrell
Zhu et al. (2004) found a three to six-fold increase in esterase activity, using \( \alpha \)- and \( \beta \)-naphthyl acetate substrates, in a population of *L. lineolaris* with an eleven-fold resistance to malathion. Zhu et al. (2011) showed a 5.3-fold increase in esterase activity and two-fold increase in glutathione s-transferase activity in field populations of *L. lineolaris* when compared to an organophosphate susceptible colony. Zhu et al. (2012) found a two-fold increase in esterase activities in a *L. lineolaris* colony with an acephate resistance ratio of 5.9. Zhu and Luttrell (2012) found up to a 5.3-fold increase in esterase activities in *L. lineolaris* that had up to a 4.3-fold resistance to acephate when compared to a susceptible colony. Zhu et al. (2007) found a 1.5-fold increase in glutathione s-transferase activity in malathion resistant strains of *L. lineolaris*. Zhu and Snodgrass (2003) found that a cytochrome P-450 gene mutation and a 2.1-fold up-regulation of cytochrome P-450 mRNA occurred in a permethrin resistant strain of *L. lineolaris*. Zhu et al. (2012) found up-regulation (>2-fold) of three cytochrome P-450 genes in an acephate resistant *L. lineolaris* colony. Zhu and Luttrell (2015) found five up-regulated (>2-fold) P-450 genes in an imidacloprid-selected *L. lineolaris* colony. Taken together, these data indicate that significant up-regulation of detoxification enzymes and their genes occurs when insecticide tolerance increases.

The purposes of our study are to compare insecticide tolerance and insecticide detoxifying enzyme activities in Delta and Hills populations of *L. lineolaris*. Based on economic data, previous research, and the lack of knowledge about the dissimilarity between regional impacts to cotton by *L. lineolaris*, experiments were undertaken to evaluate the susceptibility of acephate, imidacloprid, and permethrin, as well as, the activity of glutathione s-transferase (GST), esterase, and cytochrome P-450 in these
populations. Finding differences in insecticide resistance and detoxification enzymes will help support the theory that insecticide resistance selection is the reason for the increased difficulties of controlling *L. lineolaris* populations in Delta cotton fields.
The delta (alluvial plain) is the Delta region referred to in this paper, while the Hills refers to the rest of the state. The primary cotton growing areas of the Hills region are the areas east of the loess bluffs and north of the pine hills. * indicates counties of collection sites.
Figure 4.2  Estimated yearly insecticide applications for *L. lineolaris* on cotton in the Delta and Hills regions of Mississippi.

Trend line equations: Delta 1986 to 1999, no. of applications= -193.4+0.098(year); Delta 2000 to 2014, no. of applications= -595.4+0.299(year); Hills 1986 to 2001, no. of applications= 106.5-0.053(year); Hills 2002 to 2014, no. of applications= -416.7+0.208(year). Data taken from Williams (1986-2014) and piece-wise regression equations estimated using Proc NLIN in SAS 9.3.

**Materials and methods**

**Insect rearing**

Colonies of *L. lineolaris* were collected from wild host plants at five locations (one location per week per region) (Figure 4.1) in both the Delta and Hills regions of Mississippi during the late Spring (May 15 to June 15) of 2014. Insects were collected with sweepnets, placed into plastic containers, and aspirated into cricket cages for transport back to the Mississippi State University Insect Rearing Center. Insects were then emptied into 8.3 liter plastic containers filled with shredded paper. A fine mesh
screen was placed on the containers and was held down with the self-sealing lids of these containers which are modified with the center of the lid removed. Screen covers and shredded paper were replaced weekly to reduce contamination from fecal material. Insect colonies were kept in a rearing chamber maintained at 26.7 ± 2° C at a relative humidity of 65 ± 5% with a 16:8 light: dark cycle. The insects were fed an oligidic artificial diet similar to that of Cohen (2000), presented in a 5 x 5 cm Parafilm (Beemis Company Inc., Neenah, WI) packet placed on top of the screens. Oviposition packets consisting of a 4% carrageenan solution presented in a 5 x 5 cm Parafilm packet were also placed on top of the screens. Oviposition packets containing eggs were transferred three times weekly into 8.3 liter plastic containers similar to those housing the parent colonies. These containers remained in the same rearing chamber as the parent containers and F1 generation nymphs were reared on artificial diet to fourth instar for use in assays.

**Insecticide assays**

A glass-vial bioassay, based on the methods of Snodgrass (1996b), was used to determine LC50s for acephate, permethrin, and imidacloprid. Technical grade acephate and permethrin (Chem Service, West Chester, PA) were dissolved and diluted in acetone to make the doses for the experiment. Twenty milliliter scintillation vials were treated with doses of acephate (1.875, 3.75, 7.5, 15, 30, 60, 120 μg/vial) and permethrin (1.25, 2.5, 5, 10, 20, 40, 80 μg/vial) by pipetting 500μl of solution into the vials and rolling the vials on a repurposed Star® brand hot dog roller grill (heating element removed) until the acetone had evaporated. Cut pieces of green beans (~0.2 g) were placed in the vials to serve as a food source for the insects. Two 4th instar nymphs were placed in each vial by gently scooping them from the sides of the rearing container into the vial. A cotton ball
was placed in the mouth of the vial to prevent the nymphs from escaping. Vials were placed into the same rearing chamber as the colonies and mortality was recorded after 24 hours. Technical grade imidacloprid (Chem Service, West Chester, PA) was dissolved in acetone and diluted in honey water to make the doses (0.022, 0.067, 0.2, 0.6, 1.8 µg/cap) for the experiment. 300 µl of solution was placed into caps of 1.5 ml SealRite® 1.5 ml micro-centrifuge tubes (USA Scientific, Inc., Ocala, FL) that had been removed from the tube. Caps were then covered in Parafilm by pressing the Parafilm over the lip of the cap with a lid from an ultra-fine tip Sharpie® marker. Filled caps were placed lip side up in 20 ml scintillation vials. Two fourth instar nymphs were transferred into each vial by gently scooping the nymphs into the vials from the sides of the container. A cotton ball was placed in the top of the vial to prevent the nymphs from escaping. Vials were placed into the same rearing chamber as the colonies and mortality was recorded after 72 hours. Each treatment was replicated three times with ten nymphs per replicate.

**Glutathione s-transferase activity**

Insects from the rearing colonies were used to test for GST activity. Three replicates of twelve insects each were taken from each colony and replicates were divided into four sub-samples of three insects each. Sub-samples of insects were homogenized in 500µl of a 100 mM potassium-phosphate homogenizing buffer (approximately 2:1 dibasic:monobasic to obtain a pH 7.0) containing 2 mM EDTA disodium salt (Thermo Fisher Scientific, Waltham, MA) and 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO). The homogenate was centrifuged at 3000 g [rcf] for 5 minutes and supernatant removed and re-centrifuged at 10,000 g [rcf] for 15 minutes. Supernatant was then diluted 10:1 in a 0.1 M potassium-phosphate assay buffer containing 0.1 % Triton X-100.
20 µl of diluted sample supernatant followed by 150 µl of assay buffer and 20 µl of L-glutathione reduced (GSH) (Sigma-Aldrich, St. Louis, MO) were pipetted, in triplicate, into wells of a 96-well Fisher brand clear, flat-bottom plate. Six wells (First three wells and last three wells) on each plate were used for 170 µl of assay buffer and 20 µl of GSH that served as a negative control. 10 µl 1-chloro-2,4-dinitrobenzene (CDNB) (Sigma-Aldrich, St. Louis, MO) was added to all wells with a multi-channel pipette. Plates were placed in a BioTek Synergy H1 Multi-mode plate reader (BioTek, Winooski, VT), shaken for two seconds orbitally, and absorption was read every minute for ten minutes at 340 nm. Absorbance was calculated by subtracting the absorbance per minute of the negative control from the absorbance per minute of the samples, then dividing by the extinction coefficient of 0.00503 (Anonymous 2014) and multiplying by the dilution factor of 10 to give nmol of enzyme per minute per ml. The protein quantity of each sample was estimated using the BCA method (Smith et al. 1985). Glutathione s-transferase activity is expressed as nmol GSH conjugated per minute per mg protein (nmol/min/mg protein).

**Esterase activity**

Homogenate supernatant in assay buffer from the GST activity experiment was used to estimate esterase activity. Homogenate supernatant was vortexed, then centrifuged at 10,000 g [rcf] for 5 min. Solutions of 0.113 M α-naphthyl acetate (Sigma-Aldrich, St. Louis, MO), 0.113 M β-naphthyl acetate (Sigma-Aldrich, St. Louis, MO), and 0.06 % Fast Blue B salt (Sigma-Aldrich, St. Louis, MO) solutions were each prepared in a 0.1 M potassium-phosphate assay buffer (approximately 2:1 dibasic:monobasic to obtain a pH 7.0) containing 0.1 % Triton X-100. Substrate for the
assay was prepared by making a 2% solution of α-naphthyl acetate and β-naphthyl acetate in the Fast Blue B salt solution (FBB). Substrate solution was vacuum filtered before use. Assays were done on a Fisher brand plate like those used in the GST assay. 10 µl of sample supernatant was pipetted in triplicate into wells of the plate followed by 240 µl of substrate (either α- or β-naphthyl acetate in FBB). Each substrate was assayed on separate plates. Six wells (first three and last three) on each plate were used as a negative control which contained 10 µl of assay buffer in the place of sample supernatant. Plates were placed on the same plate reader used for the GST assay and the program was set up the same, except absorbance was set to be read at 450 nm. Protein estimates used were those previously calculated from the GST assay. Absorbance was calculated by subtracting the absorbance per minute of the negative control from the absorbance per minute of the samples and multiplying by the dilution factor of 10 to give change in absorbance per minute per ml. Esterase activity is expressed as change in absorbance per minute per mg of protein (ΔAbs/min/mg protein).

**Statistics**

Analyses of insecticide assays were conducted in SAS 9.3 (SAS Institute Inc., Cary, NC) using the Proc Probit procedure to determine LC$_{50}$s. LC$_{50}$s of each colony were considered significantly different if confidence intervals did not overlap (Table 4.1). Regional comparisons were attempted but the data indicated a lack of fit (Pearson’s Chi-square, probability<0.05). Proc Glimmix was used to compare the average LC$_{50}$s from each region. Analyses of detoxification enzyme levels were performed with the Proc Glimmix procedure of SAS 9.3. Region and location within a region (county) were independently analyzed as main effects and subsample within replicate, replicate within
county, and subsample by replicate within county were used as random terms. Degrees of freedom were calculated using the Kenward-Roger method (Kenward and Roger 1997). Differences were considered significant at $\alpha \leq 0.05$ (Fisher’s LSD).

**Results**

**Insecticide assays**

Analyses to calculate LC$_{50}$s revealed differences between counties for all three insecticides tested (Table 4.1). Differences from the highest to lowest LC$_{50}$s were 3.1-fold for acephate, 6.3-fold for imidacloprid, and 7.1-fold for permethrin. The Washington County colony had significantly higher LC$_{50}$s for acephate and permethrin than those collected from the other counties (Table 4.1). Analysis of the regional differences revealed that overall, Delta and Hills LC$_{50}$s were not different for acephate ($df=1, 8; F=0.39; p=0.55$), imidacloprid ($df=1, 8; F=0.74; p=0.41$), or permethrin ($df=1, 8; F=1.47; p=0.26$).
Table 4.1  Probit analyses and LC$_{50}$ estimates of adult *L. lineolaris* populations in Mississippi during 2014.

<table>
<thead>
<tr>
<th>Insecticide</th>
<th>Region</th>
<th>County</th>
<th>Goodness of Fit (Pearson) value</th>
<th>prob&gt;Chi</th>
<th>LC$_{50}$ 95% fiducial limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acephate</td>
<td>Delta</td>
<td>Coahoma</td>
<td>11.16</td>
<td>0.60</td>
<td>11.30 9.21 13.71 BC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Leflore</td>
<td>14.07</td>
<td>0.37</td>
<td>12.06 9.48 15.15 BC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sunflower</td>
<td>5.80</td>
<td>0.95</td>
<td>14.20 10.81 18.47 BC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Washington</td>
<td>15.37</td>
<td>0.29</td>
<td>26.25 20.89 32.72 A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Yazoo</td>
<td>9.50</td>
<td>0.73</td>
<td>8.42  6.66 10.68 C</td>
</tr>
<tr>
<td></td>
<td>Hills</td>
<td>Lowndes</td>
<td>10.19</td>
<td>0.68</td>
<td>11.53 8.54 16.55 BC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Monroe</td>
<td>12.83</td>
<td>0.46</td>
<td>15.11 11.83 19.29 B</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Oktibbeha</td>
<td>17.64</td>
<td>0.17</td>
<td>11.75  9.53 14.47 BC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Webster</td>
<td>14.22</td>
<td>0.35</td>
<td>10.19  8.19 12.75 BC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Winston</td>
<td>8.34</td>
<td>0.82</td>
<td>13.64 10.56 18.59 BC</td>
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Table 4.1 (Continued)

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<tr>
<th>Insecticide</th>
<th>Region</th>
<th>County</th>
<th>value</th>
<th>prob&gt;Chi LC50</th>
<th>95% fiducial limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imidacloprid</td>
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<td>Coahoma</td>
<td>12.23</td>
<td>0.51</td>
<td>0.16 0.08 0.25 AB</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Leflore</td>
<td>10.68</td>
<td>0.64</td>
<td>0.08 0.04 0.13 AB</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sunflower</td>
<td>8.69</td>
<td>0.80</td>
<td>0.14 0.08 0.22 AB</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Washington</td>
<td>6.87</td>
<td>0.91</td>
<td>0.11 0.07 0.17 AB</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Yazoo</td>
<td>5.56</td>
<td>0.96</td>
<td>0.25 0.10 0.46 A</td>
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<tr>
<td></td>
<td>Hills</td>
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<td>4.35</td>
<td>0.99</td>
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<td></td>
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<td>0.52</td>
<td>0.18 0.10 0.29 AB</td>
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<td></td>
<td></td>
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<td>0.04 0.02 0.07 B</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Webster</td>
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<td>0.52</td>
<td>0.10 0.04 0.21 AB</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Winston</td>
<td>3.17</td>
<td>1.00</td>
<td>0.15 0.09 0.23 AB</td>
</tr>
<tr>
<td>Permethrin</td>
<td>Delta</td>
<td>Coahoma</td>
<td>16.81</td>
<td>0.21</td>
<td>13.50 10.01 19.04 BC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Leflore</td>
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<td>11.53 8.39 16.14 BC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sunflower</td>
<td>13.79</td>
<td>0.39</td>
<td>11.14 8.99 13.73 BC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Washington</td>
<td>10.44</td>
<td>0.66</td>
<td>42.52 29.83 72.85 A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Yazoo</td>
<td>6.28</td>
<td>0.94</td>
<td>11.90 9.35 15.26 BC</td>
</tr>
<tr>
<td></td>
<td>Hills</td>
<td>Lowndes</td>
<td>18.42</td>
<td>0.14</td>
<td>9.76 7.72 12.80 BC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Monroe</td>
<td>12.85</td>
<td>0.46</td>
<td>8.74 6.64 11.32 C</td>
</tr>
<tr>
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<td></td>
<td>Oktibbeha</td>
<td>8.83</td>
<td>0.79</td>
<td>12.57 9.04 20.95 BC</td>
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<td>5.95 4.71 7.55 C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Winston</td>
<td>14.57</td>
<td>0.34</td>
<td>15.27 11.83 20.41 B</td>
</tr>
</tbody>
</table>

Degrees of freedom = 13 for all colonies and all insecticides. Rows within the same insecticide treatment followed by the same letter are not significantly different based on overlapping confidence intervals.

Glutathione s-transferase activity

Region did not have a significant effect on GST activity (df= 1, 26.37; F= 3.48; p=0.07), although the data indicate a strong trend for higher GST activity in Delta collected *L. lineolaris*. GST activity was significantly affected by the location (county) the insects were collected from (df= 9, 18.22; F=2.53; p= 0.04). GST activity ranged
from 2.27 to 3.83 nmol conjugated GSH per min per mg protein, a 1.7-fold difference (Figure 4.3).

Figure 4.3  Mean glutathione s-transferase activity in Mississippi *L. lineolaris* colonies during 2014.

Columns containing the same letter are not significantly different (Fisher’s LSD, α=0.05). N=3 for all colonies

**Esterase activity**

Region had a significant effect on esterase activity for both α-naphthyl acetate substrate (df=1, 27.06; F=11.77; p<0.01) and β-naphthyl acetate substrate (df=1, 25.85; F=10.77; p<0.01). Esterase activity was approximately 1.3-fold higher in the Delta
collected colonies than the Hills colonies for both the α- and β-naphthyl acetate substrates (Figure 4.4). Esterase activity was also significantly affected by the county of collection for both α-naphthyl acetate substrate (df=9, 99; F=6.29; p<0.01) and β-naphthyl acetate substrate (df=9, 18.1; F=3.84; p<0.01). Esterase activity was highest in the Washington county colony for both α- and β-naphthyl acetate substrates and was approximately 2-fold higher than the county with the lowest esterase activity for both substrates (Figure 4.5).

Figure 4.4 Mean and standard error of esterase activity in Mississippi L. lineolaris colonies during 2014.

Columns representing the same substrate containing the same letter are not significantly different (Fisher’s LSD, α=0.05). N=15 for all treatments.

**Discussion**

The insecticide assay data indicated that there is not a significant difference in LC$_{50}$s between the Delta and Hills regions for any of the insecticides; however, approximately three- to seven-fold differences occurred between colonies. Overall, these data show that variation occurs in insecticide tolerance in L. lineolaris populations from Mississippi and some populations in the Delta exhibit a much higher tolerance. This variation is in agreement with much of the Mississippi L. lineolaris insecticide resistance
work that has been previously conducted (Snodgrass 1994, Snodgrass 1996a, Snodgrass and Scott 2002, Zhu and Luttrell 2012, 2015) (Fred R. Musser., Mississippi State University, unpublished data).

Figure 4.5  Mean and standard error of esterase activity by county in Mississippi *L. lineolaris*.

Columns representing the same substrate containing the same letter are not significantly different (Fisher’s LSD, $\alpha=0.05$). N=3 for all treatments.

The range of LC$_{50}$s in these data are not as large as some data that has been reported (Snodgrass 1994, Snodgrass 1996a); however, small changes in insecticide tolerance can be enough to make an insect population difficult to control. According to
Snodgrass et al. (2009) a resistance ratio of 3.6 was high enough to make a field population of *L. lineolaris* unmanageable with acephate. Populations with resistance ratios lower than this may be managed but not as effectively or economically as a susceptible population. In other words, small increases in *L. lineolaris* insecticide tolerance could create populations that are not well managed with economic or labeled rates of insecticides. Based on our data, the Washington County colony could likely be difficult to manage with pyrethroids and organophosphates and the Yazoo county colony may be difficult to control with neonicotinoids.

The detoxification enzyme data indicate that esterase activity is significantly higher in *L. lineolaris* from the Delta and there was a strong trend for higher GST activity in Delta populations. There are also significant differences in populations for GST and esterase activity in those from the Delta region. However, for those colonies collected from the Hills region, esterase activity was not significantly different (Figure 4.6). The regional differences in esterase activity and lack of differences in esterase activity between Hills colonies suggests that the selection pressure in the Delta region has been different than the selection pressure in the Hills.

The esterase system has been shown to be critical in detoxifying organophosphate compounds in *L. lineolaris* (Zhu et al. 2004, Zhu et al. 2011, Zhu et al. 2012, Zhu and Luttrell 2012), as well as detoxifying pyrethroids and carbamates in other insects (Devonshire and Moores 1982, Gunning et al. 1991, Valles 1998). These three classes of insecticides are the three major classes that have been used for control of insect pests in cotton in both the Delta and Hills regions of Mississippi. The Hills, however, has not been subjected to as much insecticide usage as the Delta. The Delta is comprised of
approximately 55% row crop agriculture land while the Hills is comprised of 20% (Fry et al. 2011). This difference likely contributes to the increased resistance selection in the overall Delta *L. lineolaris* population. The large proportion of non-row crop agriculture land in the Hills provides *L. lineolaris* populations a large amount of acreage that could serve as non-sprayed refuges that would limit the amount of insecticide resistance selection, thus individuals surviving exposure to insecticides have more opportunities to mate with non-exposed individuals in the Hills region while in the Delta, there is a much smaller refuge population available to mate with the resistant individuals.

Correlations were conducted to determine if a relationship between enzyme activities and LC$_{50}$ data existed. Overall, a relationship could not be identified; however, the Washington County colony stands out as the most tolerant to acephate and permethrin while also having the highest GST and esterase activities. These data taken together with the enzyme activity data indicate that approximately a 1.4-fold increase in GST activity and 1.5-fold increase in esterase activity has led to approximately a 2.2-fold increase in acephate LC$_{50}$s and 3.7-fold increase in permethrin LC$_{50}$s in this colony. Furthermore, it seems that small amounts of up-regulation of detoxifying enzymes can potentially lead to larger increases in insecticide resistance, and has been reported in other research (Zhu et al. 2004, Zhu and Luttrell 2012). Based on our data, detoxifying enzymes seem to be a less variable and more sensitive method for detecting differences in insecticide tolerance, thus it may be important for future insecticide tolerance monitoring to include enzyme assays to get a better understanding of when a shift toward resistance is actually occurring. Taken together, our data indicate that *L. lineolaris* in the Delta would be more
difficult to manage with pyrethroid and possibly neonicotinoid insecticides, and this is likely a part of the reason that *L. lineolaris* is a more serious pest in Delta cotton.

Our data showed that the activity of metabolic enzymes was different between the two regions; however, target site resistance, specifically acetylcholine esterase inhibition insensitivity, may also be a factor. Acetylcholine esterase (AChE) insensitivity and higher activity has been shown to exist in organophosphate-resistant *Lygus* populations (Zhu and Brindley 1990, Xu and Brindley 1993, Zhu et al. 2011), and multiple resistance between esterase metabolism and AChE insensitivity may exist (Xu and Brindley 1993). Our data did not take into account any resistance mechanisms other than metabolic detoxification, thus more research is needed to determine all the mechanisms that may be contributing to *L. lineolaris* resistance, how they may vary within and between each region, and how the modes of resistance may interact.

Esterase and GST activity was not significantly up-regulated in the Yazoo county colony, indicating that these enzymes are not as important in detoxification of neonicotinoids as they are for pyrethroid and organophosphate compounds. Detoxification of neonicotinoid compounds has been shown to be related to the cytochrome P450 system (Puinean et al. 2010a, b). Attempts were made to assay cytochrome P-450 enzymes but were unsuccessful. This prevented comparisons of the cytochrome P-450 system in our colonies which may have supported the LC$_{50}$ data in the imidacloprid assay, as well as, further supporting the acephate and permethrin assays.

The reason for the discrepancy between the LC$_{50}$ data and the enzyme activity data is unknown. A possible reason for this may be due to unidentified natural variation. According to Robertson et al. (1995), natural variation may lead to erroneous conclusions.
if sources of variation are not dealt with. Unknown natural variation may explain the discrepancy in our data, but more research is needed to identify sources of natural variation in insecticide bioassays.

In summary, insecticide LC\textsubscript{50}s were not significantly different between regions for any of the insecticides tested. The variation that occurred in LC\textsubscript{50} data made it difficult to make comparisons of regional insecticide tolerance. Activity of GST and esterases indicates that a shift toward insecticide resistance has occurred in the Delta and this may be an important contributing factor to the increased damage and difficulty of control of \textit{L. lineolaris} in Delta cotton fields. Detoxification enzyme activities may be a more accurate method of detecting differences in insecticide tolerance between populations.

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CHAPTER V
A BRIEF NOTE ON THE EFFECTS OF DELTA AND HILLS POPULATIONS OF
LYGUS LINEOLARIS NYMPHS CAGED ON COTTON

Abstract

Lygus lineolaris (Palisot de Beauvois), tarnished plant bug, is a serious pest of cotton (Gossypium hirsutum L.) in Mississippi but is more difficult to manage in the Delta region of the state. In 2014, cage studies were conducted to compare damage and abscission of cotton fruiting structures in cages infested with L. lineolaris from either the Delta or Hills regions. The amount of damage or abscission that occurred was much lower than expected in comparison to uninfested cages. Abscission rates were not different between Delta and Hills cages or the uninfested cages. The percent of damaged squares and flowers was significantly higher in Hills cages than in Delta cages. The total amount of damage (both damaged and abscised positions) was not different between regions.

Introduction

L. lineolaris (Palisot de Beauvois) is the most economically important insect pest of cotton (Gossypium hirsutum L.) in Mississippi. The feeding of L. lineolaris on cotton can result in aborted or damaged squares or dirty flowers, all of which can have serious effects on the final yield if the L. lineolaris populations are above economic thresholds or
are unmanaged. Damage by *L. lineolaris* in Mississippi is not consistent throughout the state. According to the cotton insect losses (Williams 1986-2014), *L. lineolaris* costs producers in the Mississippi Delta region approximately 3.6-fold more from both insecticide costs and yield losses than the growers in the Hills region. Also, research has shown that there may be some populations better adapted to feeding on cotton. Adams et al. (2014) found that a Delta colony of *L. lineolaris* developed to adulthood 1.67 days faster than a Hills colony. This same population also laid 1.09 more eggs per day than the Hills colony. However, additional research with more colonies and additional diets did not reveal regional differences (Fleming et al. 2015).

Besides the aforementioned laboratory studies comparing feeding on cotton, no other studies are known to have been published comparing population impacts of *L. lineolaris* on cotton. Based on the economic dissimilarities between regions and the inconsistency between the two laboratory studies, a field cage study was conducted to compare the damage to cotton of *L. lineolaris* colonies from the Delta and Hills region. This chapter briefly details the results of this study.

**Materials and methods**

Cotton (Deltapine 0912) was planted on 06 June and 18 June 2014 at the Mississippi State University R.R. Foil Plant Science Research Farm in Starkville, MS. Fields were sampled for *L. lineolaris* approximately one week before infestation to ensure that natural populations were not above threshold, and this revealed natural populations to never be more than 1/4th of recommended treatment threshold (Catchot et al. 2015). Field cages, 1.8 by 1.8 by 1.8 m, were placed over two rows of cotton the day before infestation. Plant stands in each cage were reduced to 21 plants per cage for
uniformity. Infestations were made on six different days during the first two weeks of bloom for each planting date. In the earlier planted field, infestations were made on 30 July, 1 August, 4 August, and 7 August. In the later planted field, infestations were made on 15 August and 21 August. Each infestation date consisted of an equal ratio of cages infested with Delta colonies, Hills colonies, and cages not infested.

The colonies of *L. lineolaris* used in the experiments in Chapter 4 of this dissertation were used in this study. These colonies had been collected from wild hosts in the Delta and Hills regions. First generation fourth and fifth instar nymphs from these colonies were removed by gently scooping them into Solo® brand 1 1/4 oz Soufflé portion cups (five insects per cup), and sealing them in with the lids of the cups. The cups were placed into a cooler for transport to the field. Infestations were done before 9 am to limit the stress of abrupt temperature changes. Cups were tied with flagging tape near the lowest fruiting branch on the plant, and after all the cups had been tied to the plants the lids were removed. Rating of fruiting structure damage and abscission was conducted seven days after infestation. Ratings were conducted by observing the type of fruiting structure, presence or absence of structures, and damage to structures for the first two positions of all fruiting nodes on ten randomly selected plants in each cage.

Statistical analyses were conducted using the Proc Glimmix procedure of SAS 9.3 (SAS Institute, Cary, NC). Data analyzed were the percent of healthy positions (present and not damaged versus abscised or damaged), percent of present positions versus abscised positions, and the percent of healthy versus damaged positions for those present (excluding abscised positions). Initial analyses revealed variation with infestation date and the individuals conducting the ratings. Terms to account for variation of the person
conducting the rating, infestation date, replicate, and colony were used in the random statement. Degrees of freedom were calculated using the Kenward-Roger method (Kenward and Roger 1997). Differences were considered significant at $\alpha \leq 0.05$ (Fisher’s LSD).

**Results**

There was not a significant difference in the percent of abscised positions ($df=2$, 1.11; $F=2.33; p=0.4$) between Delta and Hills colonies or between colonies from either region and the uninfested cages (Figure 5.1). Cages from both regions had significantly more damage to fruiting structures (squares and blooms) ($df=2$, 6.18; $F=12.34; p<0.01$) than the uninfested cages and *L. lineolaris* in Hills colony cages caused more damage than those in Delta colony cages (Figure 5.1). The total amount of damage (abscission and damaged fruit) was significantly higher ($df=2$, 28.89; $F=12.64; p<0.01$) in infested cages than the uninfested cages, but the Delta colonies were not different from the Hills colonies (Figure 5.1). Overall, these data show that more damage occurred in the infested cages than in the uninfested cages, which was primarily caused by damage to positions not abscission.

**Discussion**

Our data revealed that the Hills population of *L. lineolaris* may be more damaging to cotton than the Delta population in regards to the percent of damaged squares and flowers. However, the level of damage and abscission that occurred in the infested cages when compared to the uninfested cages was lower than expected. Our data revealed approximately 10 % more abscission than in uninfested cages and approximately 15 %
more damaged fruit in infested cages. Our rate of infestation was approximately 29 insects per row m (8.75 per row ft). This rate is approximately 17.5 times higher than the threshold of three per 1.8 row m (three per six row ft) (Catchot et al. 2015).

Figure 5.1  Mean and standard error of the percentage of damage and abscission to caged cotton by *L. lineolaris* collected from the Delta and Hills regions of Mississippi during 2014.

Columns within the same variable sharing the same letter are not significantly different (Fisher’s LSD, α=0.05). N=13 for all variables.

In other cage studies, square abscission has been shown to be in the range of 0.6 to 2.1 abscised squares per insect per day (Wilson 1984). Based on these data, even at the lower rate of abscission, there should have been enough insects per cage to cause abscission or damage of almost all the squares in the cage. The reason for the lower than
expected damage and abscission in our study is unknown, but not totally unexpected. For example, Musser et al. (2009) was able to show that natural *L. lineolaris* populations could significantly impact the yield of cotton when feeding during the flowering period, while work by Scales and Furr (1968), Jubb and Carruth (1971), and Tugwell et al. (1976), using released populations and caged plants, did not show significant impacts of *L. lineolaris* to flowering cotton. Additionally, it is widely recognized by other researchers that cage studies are not always successful or yield unexplainable results. Additional work using natural populations, which have not been kept under artificial rearing conditions, may be necessary to compare Delta and Hills populations to determine if the two populations differ in their abilities to damage cotton.

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References


CHAPTER VI
CONCLUSION AND FUTURE CONSIDERATIONS

Why is *Lygus lineolaris* a More Serious Pest in Delta Cotton?

Based on our data, the most likely contributing factor for the higher costs of managing *L. lineolaris* in the Delta is related to up-regulation of detoxifying enzymes (increased metabolic resistance) in the Delta populations. This is not surprising, as insecticide resistance has been recognized in Delta populations for many years. What is surprising is that data for LC$_{50}$S, in both our study as well as the studies of previous authors, was too variable to show regional differences. The reason for the variability is unknown but is likely caused by a combination of ecological and biological factors that are not fully understood. Future insecticide resistance work should include enzyme assays that may detect differences where insecticide assays may lack the sensitivity to determine differences between populations.

Our other research using biology and genetics to compare populations failed to show regional differences. This dissertation began with the hypothesis that the Delta population of *L. lineolaris* may be a biotype or sibling species that had selected for feeding and development on cotton. Based on our data we cannot confirm this hypothesis. Biological data indicated that no regional differences existed for any of the variables measured, but showed that diet has a significant effect on developmental time, fecundity, and survival. Comparisons of the polygalacturonase system revealed that this
system has not been selected for cotton in Delta populations of *L. lineolaris*. These data also revealed that specific PG genes may be more important for the transcription, and ultimately translation, of PG isoenzymes for specific hosts; however, no specific gene was found for cotton. At this time metabolic resistance seems a major contributing physiological mechanism for the increased pest pressure of *L. lineolaris* in the Delta.

This leads to the question of why metabolic resistance has occurred. Resistance evolution in the Delta populations of *L. lineolaris* has likely increased faster than that of populations in the Hills due to differences in landscapes. The Delta is a much more agriculturally intense landscape than the Hills. This results in more of the Delta landscape receiving insecticides while less of the Hills receives insecticide applications. Over time this has led to a larger proportion of the Delta population selected for insecticide detoxification. If landscapes and their relation to insecticide use are a major contributor to the development of insecticide resistance then landscapes should be seen as a tool for insecticide resistance management. However, manipulating the land use patterns in the Delta landscape in a way that would increase refuge areas for *L. lineolaris* is probably not a viable alternative

**Future directions**

1) More research is needed with the polygalacturonase system. Based on our data, there does not seem to be a specific PG gene that could be targeted with an RNAi insecticide or polygalacturonase inhibiting cotton varieties, thus a broad approach, such as targeting the enzyme or targeting multiple genes, would be needed. Cotton contains polygalacturonase inhibiting proteins (PGIP) known to inhibit fungal PG. It is likely that cotton may contain PGIP that inhibits insect PG as well. If these could be found or if
insect inhibiting PGIP genes from other plants could be transferred to cotton then these new varieties could be another tool for the IPM toolbox.

2) The relationship between insecticide LC$_{50}$ and detoxifying enzymes needs to be further explored. Future work involving resistant populations should include enzyme assays to further elucidate possible resistance mechanisms.

3) Further study is needed to determine the extent of other insecticide resistance mechanisms in $L$. lineolaris and to determine if any of these other mechanisms may contribute to the Delta and Hills differences

4) A protocol for isolating microsomal material for cytochrome P-450 assays is needed. The ability to easily assay cytochrome P-450 would give researchers the ability to better monitor neonicotinoid resistance.

5) Research is needed to explore how landscapes can impact the development of insecticide resistance. Once this is understood, research could be undertaken to determine ways the landscape could be changed to slow resistance to current insecticides and prevent resistance to novel chemistries.

6) Another area that may be contributor to the higher pest pressure of $L$. lineolaris on cotton in the Delta is movement in relation to landscapes. The phenology of the movement of $L$. lineolaris into cotton is not fully understood, but because of its polyphagous nature, other crops and wild hosts have a role. Host plant species compositions and the physical makeup of the Delta is different than the Hills. The availability of nursery hosts as well as the physical barriers to movement (trees and hills vs crops and flat land) may affect movement and should be studied to determine if, for example, it is simply easier for $L$. lineolaris to move into cotton fields in the Delta. This
could lead to better management of landscapes to prevent severe *L. lineolaris* populations.

In conclusion, metabolic insecticide resistance seems to be a major factor in the more severe pest pressure of *L. lineolaris* in the Delta. This resistance has likely occurred because of the intensively managed agricultural landscape of the Delta region. Further research needs to be conducted to elucidate the possibilities of RNAi insecticides and PGIP cotton as control tactics for *L. lineolaris*. Also, landscapes need to be further studied to determine ways to slow or prevent insecticide resistance, while also making movement of *L. lineolaris* into cotton more difficult.