

# Relationships Among the Feeding Behaviors of a Mirid Bug on Cotton Leaves of Different Ages and Plant Biochemical Substances

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## Abstract

*Apolygus lucorum* (Meyer-Dür) (Hemiptera: Miridae) tends to feed on young plant tissues. To explore the relationship between stylet probing behaviors of adult *A. lucorum* and conditions of cotton leaves, we conducted an experiment using electropetrography (EPG). Behaviors were recorded on four cotton varieties, in relation to thickness and biochemical traits of differently-aged leaves. Cotton leaf age had a significant effect on the probing behavior of *A. lucorum* but cotton variety did not. One-day-old leaves of *A. lucorum* received the highest mean number of stylet probes (penetrations) per insect, and longest mean durations per insect of combined stylet probing or its components, cell rupture and ingestion behaviors. All of the leaf traits (thickness and biochemical substances) were similar among these four cotton varieties. Leaf thickness had a significantly negative effect on the same four variables above. Gossypol and tannin also had a negative impact on combined probing duration. Redundancy analysis showed that the four EPG variables were closely related to nutrient substances (amino acids, sugar, and water) while they had the opposite relationship with plant defense substances (gossypol and tannin). On cotton in the seedling stages, *A. lucorum* fed more readily on the youngest, thinnest leaves in our no-choice EPG experiments. Nutrients and chemical resistance substances determined the probing duration of *A. lucorum*. Our findings can contribute to better understanding of patterns of feeding and host consumption by *A. lucorum*, ultimately improving cotton resistance to *A. lucorum*.

**Key words:** electrical penetration graph, *Apolygus lucorum*, leaf trait, biochemical substance, cotton

The mirid bug, *Apolygus lucorum* (Meyer-Dür) (Hemiptera: Miridae), is a polyphagous pest with at least 242 different host species in 49 different families (Pan et al. 2013). It is widely distributed, including in Asia, Europe, North America, and northern Africa (Zhang et al. 2018). In recent years, the large-scale promotion of genetically modified, insect-resistant cotton has enabled effective control of lepidopteran pests, mainly cotton bollworms. However, nontarget piercing and sucking pests, such as *A. lucorum*, are on the rise and have become the main cotton pests (Men et al. 2005, Lu et al. 2010, Chen et al. 2010).

Attributes of plant leaves, including physical and chemical properties on the leaf surface and inside the leaf tissue, are important criteria for insects to decide whether or not to feed on a plant (Xu et al. 1994). Physical properties of the leaves have an important

impact on plant resistance against pests. For example, the thickness of the epidermis and oily spots on foliage reduce the damage caused by *A. lucorum* (Lin et al. 2011), while trichomes resist aphids, spider mites, and leafhoppers (Lu et al. 2004, Nibouche et al. 2008). Biochemical substances (secondary metabolites and nutrients) in plants can be important antibiotic substances, which resist insects by affecting their behavior, growth, and development (Yang et al. 2013). Tang and Wang (1996) showed that total proteins inhibited the growth and development, whereas sugars promoted the growth of cotton bollworm larvae. A tannic acid concentration higher than 0.03% significantly increased the proportion of nonprobing (NP) waveforms in electropetrographic (EPG) studies of *Macrosiphum granarium* (Kirby) (Hemiptera: Aphididae) and *Rhopalosiphum padi* (Linnaeus) (Hemiptera: Aphididae), and also reduced the

proportions of waveforms representing pathway (searching), salivation, and ingestion, compared with controls (Chen 2001). The types and concentrations of biochemical substances in plants are closely related to plant growth period. Another study showed that the nutrient supply of leaves and organs at different growth stages varies greatly, affecting the oviposition and survival rate of insects and subsequently, the size of insect populations (Zhao et al. 2011a). Herein, we use ‘feeding’ to mean consumption of food in an overarching, ecological sense, whereas we use ‘stylet probing’ to mean the specific, behavioral details of food consumption. EPG often has been used to study the detailed stylet probing behaviors of aphids (e.g., Hu et al. 2013) and other piercing-sucking insects, e.g., whiteflies (Janssen et al. 1989, Johnson and Walker 1999), leafhoppers (Young 1998, Miao and Han 2008), thrips (Harrewijn et al. 1996, Kindt et al. 2003), and mirid bugs (Cline and Backus 2002, Cervantes et al. 2016, Backus et al. 2019). *Apolygus lucorum* is different from aphids, whiteflies, and other sap feeders cited above, which use the salivary sheath feeding strategy. Instead, *A. lucorum* mainly feeds on the cytoplasm and nuclei of plant cells, using the strategy of cell rupture feeding (Cervantes et al. 2016). *Apolygus lucorum* tends to feed on young plant tissues. Previous EPG studies found multiple, categorizable waveforms for *A. lucorum* feeding on cotton leaf, either four waveforms (Li et al. 2014) or six (Song et al. 2017). The latter study found that EPG waveforms occurred more on young leaves than on old leaves. However, a deeper study is needed to analyze the detailed performance of probing waveforms for *A. lucorum* feeding on cotton leaf. EPG studies of other mirid bugs, such as *Lygus lineolaris* (Palisot de Beauvois) (Hemiptera: Miridae) feeding on cotton squares, showed that probing behaviors such as cell rupturing could trigger release of tannins (Cervantes et al. 2017). This finding raises the question of how the probing behaviors of mirid bugs respond to plant chemical or physical defense. Answering this question could improve our understanding of feeding habits of mirid bugs and develop pest management tactics against this pest.

This study used EPG to measure the stylet probing behaviors of *A. lucorum* on cotton leaves of four varieties and at five leaf ages. We also investigated the relationships among stylet probing behaviors of *A. lucorum* and the morphological features and biochemical substances of cotton leaves. This study aims to clarify the probing of *A. lucorum* and to explain the plant stimuli that trigger greater acceptance for consuming tender plant material.

## Materials and Methods

### Insect Sources Used in the Study

Overwintering *A. lucorum* eggs were collected from the winter jube garden in Zhanhua district, Binzhou city, Shandong Province, China. After the overwintering eggs hatched, they were reared indoors on fresh kidney bean pods at 26°C, relative humidity 80%, and photoperiod of 16:8 (L:D) h. Female *A. lucorum* adults (3–5 d after eclosion) were selected for 1-d feeding on cotton varieties and were starved for 6 h before wiring.

### Tested Cotton Varieties

The four cotton varieties used in this study, i.e., Lumianyan 37, Lumianyan 22, Lumianyan 28, and GK-688, are commonly grown cotton varieties in the sponge production area of Huanghuai in China (south of the Yellow River and north of the Huai River), provided by the Cotton Research Center of Shandong Academy of Agricultural Sciences (Shandong Province, China). Cotton varieties were planted indoors in 15-inch diameter pots in potting soil with

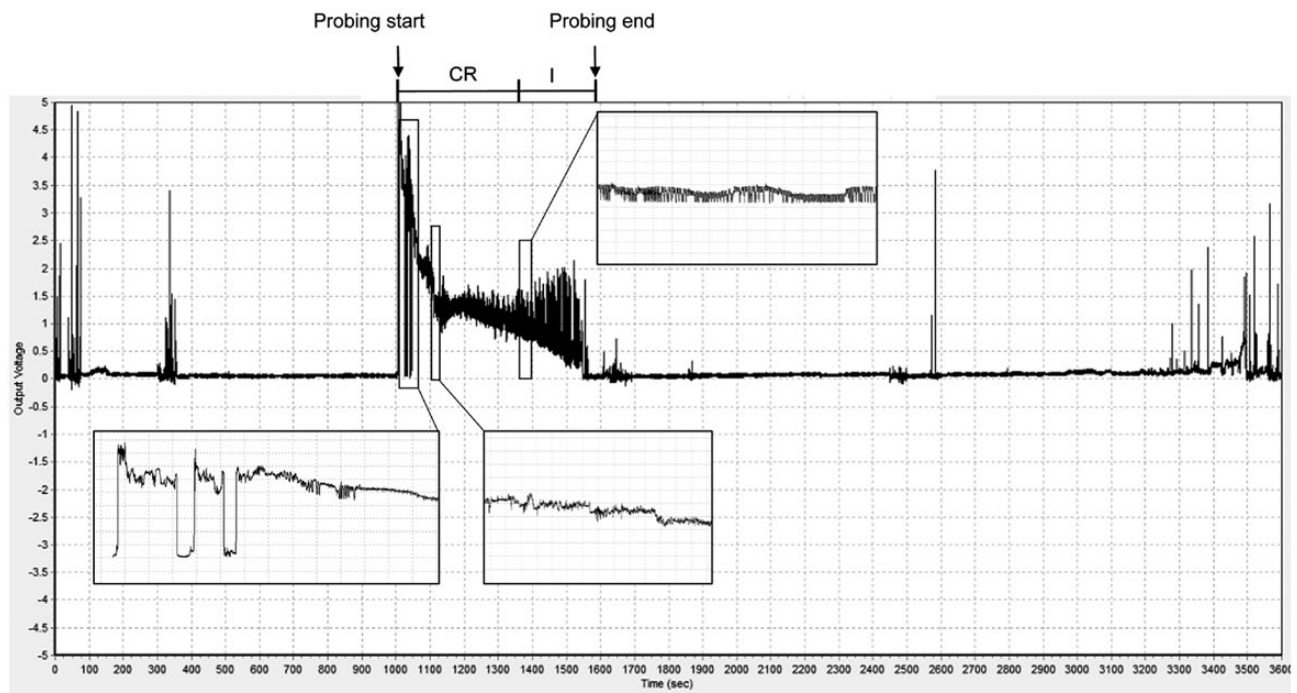
compost added. Plants were reared in greenhouses under the same conditions as above. When the first true leaf of cotton was fully expanded, it was marked as the first true leaf for testing. Marked leaves were used for tests on the same day as well as the following 5th, 10th, 15th, and 20th days when the leaves unfolded; these leaves were named 1-, 5-, 10-, 15-, and 20-d-old leaves, respectively. The same action was performed on each cotton variety.

### Electropenetrography

A Giga-8 DC EPG (EPG Systems, W.F. Tjallingii, University of Wageningen, Wageningen, The Netherlands) was used in this study. The insect electrode for insect testing consisted of a thin copper wire soldered to a brass nail, with the tip of copper wire connected to a 3-cm gold wire (diameter of 20 µm) using silver conductive glue (from EPG Systems). Each starved female *A. lucorum* (3–5 d after eclosion) was anesthetized with CO<sub>2</sub> and placed with its dorsum facing upwards. A suitable amount of conductive glue was placed on the gold wire end of the insect electrode to quickly adhere it to the end of the pronotum of the *A. lucorum*. The brass nail of the insect electrode was inserted into the corresponding slot of the head stage amplifier, followed by placing the glued *A. lucorum* on the upper surface of the cotton leaf and maintaining a certain range of space. Subsequently, a rod-shaped copper wire, approx. 10 cm long, was inserted into the potting soil of the cotton plant as the plant electrode, to connect the circuit. Data were collected and stored automatically using the ANA 34 software for waveform analysis and data sorting. Each *A. lucorum* was tested only once, and each insect was recorded continuously for 8 h. We used a randomized complete block design to cover all 20 treatments of the 4 × 5 factorial design (i.e., cotton varieties Lumianyan 37, Lumianyan 22, Lumianyan 28, and GK-688, vs leaf age levels 1, 5, 10, 15, and 20 d old). Thus, the experimental unit was replicated 20 times, giving a total of 400 insects recorded.

### Measurement of Waveforms

Waveforms were named and annotated according to a hierarchical convention (Almeida and Backus 2004, Backus et al. 2005). Probing includes all behaviors from the start of stylet insertion into plant tissue until stylet withdrawal, whereas nonprobing refers to all other behaviors that do not involve stylet penetration (Tuelher et al. 2020). The next level typically is phase; however, mirid probing is simpler than aphid probing, so there is no matching level for phase. Waveforms were measured after categorization into two different waveform families: cell rupture (CR) and ingestion (I; Fig. 1), as in Cervantes et al. (2016). CR encompasses the *A. lucorum* waveforms previously termed P (probing), B (tearing cells and salivation, including B1, B2, B3, B4 types), whereas I encompasses the previously termed S waves (ingestion, including S1, S2, and S3 subtypes; Song et al. 2017). We calculated the following four variables using the formulae and naming convention of Backus et al. (2007): 1) (mean) probing duration per insect (PDI), 2) (mean) number of probes per insect (NPI), 3) (mean) waveform duration per insect (WDI) for cell rupture, and 4) WDI for ingestion. In the present article, ‘probing duration’ refers to both families of waveforms (CR and I), combined. A ‘waveform duration’ refers to either CR or I duration. For our initial statistical tests (see below), we originally subdivided families CR and I into four and three waveform types, respectively. However, there were no significant differences among treatments for such waveform types, so subsequent tests were performed with waveforms simply divided into families CR and I.



**Fig. 1.** Representative EPG waveforms for *Apolygus lucorum* feeding on cotton leaf, using fixed input resistor of  $10^9$  Ohms and 50 mv applied signal. Boxed waveforms are enlarged in inset boxes.

### Determination of the Traits and Biochemical Substances of the Leaves

The contents of biochemical substances, including soluble sugars, tannin, gossypol, soluble proteins, and amino acids, were determined by commercially available kits (micro method) purchased from the Suzhou Comin Biotechnology Co., Ltd (Jiangsu Province, China, website: <http://www.cominbio.com/index.html> in Chinese). Each substance had its corresponding kit. Plant tissues were prepared following manufacturer's instructions. The soluble sugar, tannin, soluble proteins and amino acids were each measured separately by using enzymatic assay methods. Gossypol was measured using high-performance liquid chromatography. The measurements were repeated five times for each leaf age. Determination of the wax content on the leaf surface was performed using the chloroform method, and the experiment was repeated five times on each different measurement day. Leaf thickness was determined by excising a portion of the middle of the cotton leaf, laying it flat on a glass slide, followed by manual measurement of the leaf thickness under a light microscope at 400 $\times$  magnification. Twenty leaves, one per treatment, were measured for leaf thickness every day.

### Data Processing

Multivariate analysis of variance (MANOVA) was used to explore the overall influence of leaf age and variety of cotton plant on the probing behavior of *A. lucorum* using the four EPG variables listed above. Because the four cotton varieties did not exhibit an obvious influence on the analysis results ( $P > 0.05$ ), the different varieties were pooled for the subsequent analyses. One-way analysis of variance (ANOVA) was then used to explore the influence of leaf age for each of the four EPG variables. Multiple comparisons were performed using Fisher's least significant difference (LSD) tests. Pearson's correlation analysis was used to analyze the relationship of each of the four EPG variables with leaf indices. The function 'rda' in the 'vegan' package of the R language then was used to carry

out the redundancy analysis (RDA), to simultaneously explore the relationship of all the EPG variables with leaf traits at all leaf ages.

## Results

### Impacts of Cotton Varieties Alone on Stylet Probing

The WDIs for both cell rupture (CR) and ingestion (I) were not significantly different among the four cotton varieties (Table 1). The NPI was highest at 1 d after feeding, across all cotton varieties. PDI, as well as WDIs, for both CR and I was also the longest at 1 d. All durations significantly declined with increasing age of leaves, in a pattern that was similar among all cotton varieties (Table 1).

### Impacts of Cotton Varieties and Leaf Age on Stylet Probing

The MANOVA results showed that leaf age had a significant impact ( $df = 4.15$ ,  $P = 0.004$ ), whereas cotton variety had no significant impact on the overall probing behavior of *A. lucorum*. Despite the latter finding, the ANOVA results showed that WDI for CR was significantly different among varieties, but no other EPG waveform was. In contrast, ANOVA showed that leaf age had a significant impact on every EPG variable (Table 2). As leaf age increased, PDI and both WDIs for CR and I all decreased (Fig. 2). On 1-d-old leaves, relatively shorter WDI for CR is combined with much longer WDI for I. On 5-d-old leaves, both CR and I are reduced, but I more so than CR. At day 10, probing behavior switches; CR and I durations are no longer significantly different. On older leaves, WDI for CR is significantly lower than WDI for I (Fig. 2).

### Relationships Among Stylet Probing Behaviors and Leaf Traits

Leaf traits (leaf thickness and biochemicals) among the four cotton varieties were mostly similar within each leaf age (Table 3). Some

**Table 1.** Results for the EPG variables of *Apolygus lucorum* on four different cotton varieties and at different leaf ages

Varieties	Age (d)	Number of probes per insect	Probing duration per insect	Cell rupture duration per insect	Ingestion duration per insect
GK-688	1	4.47 ± 0.40a	2636.877 ± 201.65a	898.47 ± 55.97a	1738.40 ± 163.92a
	5	4.27 ± 0.32a	1563.00 ± 60.85b	689.33 ± 46.87b	873.67 ± 37.71b
	10	3.27 ± 0.15b	1327.27 ± 67.78c	713.80 ± 42.05b	613.47 ± 37.06c
	15	2.87 ± 0.39b	976.80 ± 93.54d	606.60 ± 72.06b	370.20 ± 32.87d
	20	3.20 ± 0.40b	631.20 ± 61.89e	410.80 ± 52.87c	220.40 ± 23.18e
Lumianyan 22	1	4.90 ± 0.39a	2818.35 ± 409.25a	838.50 ± 102.54a	1979.85 ± 326.38a
	5	3.65 ± 0.30b	1881.2 ± 200.96b	635.20 ± 66.13ab	1246.00 ± 159.85a
	10	3.40 ± 0.37b	1260.4 ± 142.41c	579.60 ± 81.38ab	680.80 ± 74.56a
	15	3.85 ± 0.55ab	825.25 ± 105.10d	467.40 ± 65.36b	357.85 ± 50.63b
	20	3.16 ± 0.67b	728.15 ± 111.82d	453.85 ± 80.58b	274.30 ± 43.97b
Lumianyan 28	1	4.40 ± 0.36a	2423.75 ± 167.85a	759.85 ± 61.66a	1663.90 ± 131.21a
	5	3.15 ± 0.24b	1474.70 ± 132.75b	605.90 ± 49.34ab	868.80 ± 101.91b
	10	3.50 ± 0.38ab	1189.35 ± 89.72c	550.15 ± 65.09b	639.20 ± 43.72b
	15	2.90 ± 0.24b	625.75 ± 73.77d	362.35 ± 45.04c	263.40 ± 35.63c
	20	2.05 ± 0.17c	436.15 ± 65.48e	257.40 ± 39.76c	178.75 ± 30.50c
Lumianyan 37	1	5.25 ± 0.62a	3389.05 ± 299.57a	1070.15 ± 77.76a	2318.90 ± 249.29a
	5	4.00 ± 0.42b	1767.7 ± 151.99b	648.90 ± 54.31b	1118.80 ± 113.93b
	10	3.60 ± 0.37bc	998.4 ± 79.01c	549.85 ± 55.26b	448.55 ± 38.06c
	15	2.85 ± 0.58bc	794.9 ± 69.85d	516.35 ± 61.40b	278.55 ± 32.11d
	20	2.50 ± 0.49c	405.35 ± 41.44e	258.10 ± 31.87c	147.25 ± 17.14e

Mean ± SE. Different lowercase letters in the same column respectively indicate significant different at  $P < 0.05$  level by LSD test.

leaf traits were found to affect probing behaviors (Table 4), especially leaf thickness, which had a significant impact on all four EPG variables of *A. lucorum*. Effects were more variable for biochemicals. Tannins had a significant impact only on PDI. Gossypol and amino acids had significant impacts on WDI for I, whereas wax, soluble sugars, and soluble proteins had no significant impact on any EPG variables (Table 4).

The RDA results (Fig. 3) showed that leaf age had a strong negative correlation with the EPG variables. In the RDA, three EPG variables, that is, PDI and both WDIs for CR and I, clustered on the negative (left) side of the first component of RDA. Amino acids, sugar, and water also were negatively correlated with the EPG variables. In contrast, tannins, leaf thickness, gossypol, and soluble proteins were on the positive (right) side (Fig. 3).

## Discussion

### Waveform Names and EPG Systems

Waveform naming conventions in EPG are diverse, depending on the underlying variability in behaviors being represented. Stylet probing behaviors of aphids and other related sternorrhynchan hemipterans such as whiteflies, psyllids, and mealybugs are quite similar; thus, their waveform naming convention has been standardized for decades. In contrast, probing behaviors and waveform appearances of auchenorrhynchan hemipterans such as leaf- and planthoppers are highly variable. Despite many decades of EPG recordings, there has not yet been published any overarching organization to their waveforms. Therefore, different authors use different names. Nonetheless, today, most waveform naming conventions for salivary sheath feeders are coalescing around a hierarchical structure within probing (from biggest to smallest categories) of phase, family, type, subtype, and (rarely) sub-subtype.

Similarly, EPG studies on mirid bugs do not have a universal naming convention. Earlier *A. lucorum* studies named waveform families (then termed phases) based on voltage patterns visible to the human eye (e.g., Zhao et al. 2011b, Song et al. 2017, Lu et al. 2020). However, some specific waveforms named therein were not

well correlated with specific probing behaviors, so the name and definition of mirid waveforms are still evolving. Fortunately, most waveform phases/families of *A. lucorum* correspond to different hierarchical categories of probing, including stylet probing/penetration, slow stylet movements combined with enzymatic maceration (together termed cell rupturing), and ingestion (fluid uptake and swallowing). In the present study, we coordinated our naming system for the same insect taxa with similar feeding strategy as those in other publications. Thus, for mirid bugs, each family during probing (e.g., cell rupture) can be labeled with capital letters (e.g., CR) according to Cervantes et al (2016).

Using the DC EPG system might have caused some problems with mirid probing behavior because this instrument uses DC applied signal combined with an input resistor ( $R_i$ ) of  $10^9 \Omega$ . When  $10^9 R_i$  is combined with high applied voltage, it can cause decreased probing and other abnormalities in mirid behavior (Backus et al. 2018). It is possible that lack of significant differences herein among EPG variables for leaf traits could have been caused by DC signal. However, the level of applied voltage we used was about 50 mv, which was not high compared to the tested voltage in Backus et al. (2018). We therefore believe that negative effects of DC applied voltage from our instrument were limited. That said, this could only be definitively determined if AC and DC applied signals were directly compared.

### Correlation of Probing With Physical and Chemical Characteristics of Cotton

The mirid bug *A. lucorum* mostly aggregates on the tender tissues of plant sprouts, leaves, buds, flowers, young fruits, and so on because they have a feeding preference for tender plant tissues (Li et al. 2013, 2014). In this study, EPG was used to study the probing behavior of *A. lucorum* on leaves of four cotton varieties at different ages. The results showed that cotton variety had no significant impact on probing behaviors of *A. lucorum*, whereas leaf age did have a significant impact. One-day-old leaves received the highest number of probes per insect and longest durations per insect of cell rupture and ingestion, i.e., *A. lucorum* tended to probe the youngest leaves of cotton plants in the seedling stage, showing greater acceptance of young, tender plant





**Table 3.** Leaf thickness and content of biochemicals in cotton leaves at the different ages

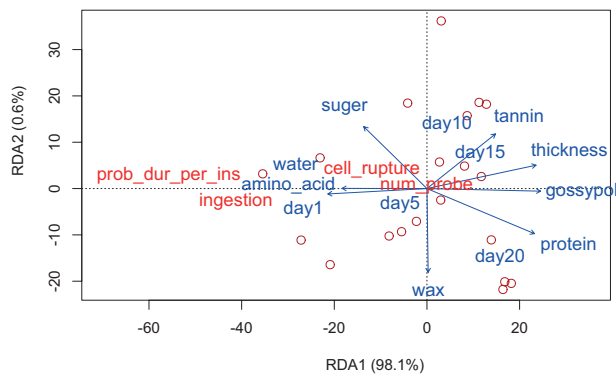
Varieties	Age	Thickness ( $\mu\text{m}$ )	Waxiness ( $\mu\text{g}/\text{mg}$ )	Tannic (mg/g)	Gossypol (%)	Sugar (mg/g)	Protein (mg/ml)	Amino acid ( $\mu\text{mol}/\text{g}$ )
GK-688	1	192.72 $\pm$ 2.54b	9.33 $\pm$ 0.13a	7.08 $\pm$ 0.18c	0.17 $\pm$ 0.00b	4.79 $\pm$ 0.20a	1.06 $\pm$ 0.03b	4.59 $\pm$ 0.36a
	5	203.94 $\pm$ 2012a	9.37 $\pm$ 0.13a	7.52 $\pm$ 0.27c	0.20 $\pm$ 0.00a	4.89 $\pm$ 0.16a	1.15 $\pm$ 0.06ab	4.23 $\pm$ 0.21a
	10	203.86 $\pm$ 3.18a	9.43 $\pm$ 0.10a	8.06 $\pm$ 0.26c	0.19 $\pm$ 0.01a	3.56 $\pm$ 0.27b	1.24 $\pm$ 0.09ab	2.14 $\pm$ 0.52b
	15	203.81 $\pm$ 2.58a	9.67 $\pm$ 0.17a	9.06 $\pm$ 0.30b	0.19 $\pm$ 0.00a	2.82 $\pm$ 0.17bc	1.26 $\pm$ 0.03a	2.08 $\pm$ 0.21b
	20	205.63 $\pm$ 2.37a	9.49 $\pm$ 0.19a	10.68 $\pm$ 0.51a	0.20 $\pm$ 0.01a	2.37 $\pm$ 0.34c	1.32 $\pm$ 0.07a	1.47 $\pm$ 0.29b
Luminyan 22	1	186.64 $\pm$ 4.09b	11.15 $\pm$ 0.24a	4.77 $\pm$ 0.19c	0.13 $\pm$ 0.01c	2.49 $\pm$ 0.17a	0.99 $\pm$ 0.06b	4.84 $\pm$ 0.44ab
	5	199.51 $\pm$ 2.38a	11.96 $\pm$ 0.28a	5.49 $\pm$ 0.20b	0.19 $\pm$ 0.00b	2.63 $\pm$ 0.20a	1.19 $\pm$ 0.06ab	5.25 $\pm$ 0.33a
	10	203.13 $\pm$ 2.16a	11.69 $\pm$ 0.23a	6.16 $\pm$ 0.22a	0.15 $\pm$ 0.01c	1.93 $\pm$ 0.65b	1.17 $\pm$ 0.05ab	4.33 $\pm$ 0.40b
	15	201.44 $\pm$ 2.63a	12.03 $\pm$ 0.23a	6.36 $\pm$ 0.29a	0.21 $\pm$ 0.00a	1.04 $\pm$ 0.88c	1.34 $\pm$ 0.16ab	2.67 $\pm$ 0.25b
	20	205.63 $\pm$ 1.65a	12.02 $\pm$ 0.28a	6.57 $\pm$ 0.28a	0.25 $\pm$ 0.01a	0.73 $\pm$ 0.87c	1.59 $\pm$ 0.18a	1.27 $\pm$ 0.22c
Luminyan 28	1	182.27 $\pm$ 3.72c	12.32 $\pm$ 0.28a	4.47 $\pm$ 0.22b	0.11 $\pm$ 0.01c	2.23 $\pm$ 0.19a	1.06 $\pm$ 0.23b	4.57 $\pm$ 0.42a
	5	196.91 $\pm$ 2.01b	12.41 $\pm$ 0.30a	5.44 $\pm$ 0.21b	0.16 $\pm$ 0.01b	2.38 $\pm$ 0.16a	1.18 $\pm$ 0.08b	4.67 $\pm$ 0.21a
	10	199.01 $\pm$ 3.49ab	12.56 $\pm$ 0.27a	6.10 $\pm$ 0.31ab	0.18 $\pm$ 0.01b	0.95 $\pm$ 0.13b	1.30 $\pm$ 0.11ab	2.57 $\pm$ 0.25b
	15	201.76 $\pm$ 3.74ab	12.58 $\pm$ 0.25a	6.94 $\pm$ 0.21a	0.20 $\pm$ 0.01ab	0.90 $\pm$ 0.47b	1.38 $\pm$ 0.11ab	1.78 $\pm$ 0.31c
	20	206.01 $\pm$ 2.57a	12.55 $\pm$ 0.26a	6.60 $\pm$ 0.24a	0.22 $\pm$ 0.01a	0.71 $\pm$ 0.11b	1.52 $\pm$ 0.15a	0.88 $\pm$ 0.09c
Luminyan 37	1	186.29 $\pm$ 3.81b	11.49 $\pm$ 0.18a	5.47 $\pm$ 0.50b	0.08 $\pm$ 0.01b	1.93 $\pm$ 0.25a	0.84 $\pm$ 0.03c	4.08 $\pm$ 0.50ab
	5	191.71 $\pm$ 3.11b	11.52 $\pm$ 0.24a	6.82 $\pm$ 0.44a	0.19 $\pm$ 0.00a	2.20 $\pm$ 0.12a	1.09 $\pm$ 0.03b	5.66 $\pm$ 1.00a
	10	197.05 $\pm$ 2.85ab	11.52 $\pm$ 0.23a	6.44 $\pm$ 0.58a	0.15 $\pm$ 0.02a	1.64 $\pm$ 0.12a	1.13 $\pm$ 0.06b	2.53 $\pm$ 0.53b
	15	200.02 $\pm$ 2.72ab	11.80 $\pm$ 0.27a	7.19 $\pm$ 0.47a	0.22 $\pm$ 0.16a	0.78 $\pm$ 0.11b	1.23 $\pm$ 0.06b	1.29 $\pm$ 0.09bc
	20	203.66 $\pm$ 2.31a	11.89 $\pm$ 0.20a	7.35 $\pm$ 0.22a	0.22 $\pm$ 0.01a	0.80 $\pm$ 0.16b	1.72 $\pm$ 0.12a	1.09 $\pm$ 0.22c

Mean  $\pm$  SE. Different lowercase letters in the same column respectively indicate significant different at  $P < 0.05$  level by LSD test.

**Table 4.** Influence of blade thickness and biochemical substances on the EPG variables of *A. lucorum*

	Number of probes per insect			Probing duration per insect			Cell rupture duration per insect			Ingestion duration per insect		
	F	df	P	F	df	P	F	df	P	F	df	P
	Thickness	5.44	399	0.02	2.87	399	0.04	6.45	399	0.01	14.74	399
Wax	0.04	99	0.42	0.14	99	0.35	0.02	99	0.45	0.05	99	0.41
Tannin	1.97	99	0.08	3.88	99	0.03	1.18	99	0.14	0.81	99	0.18
Gossypol	0.89	99	0.17	1.16	99	0.14	1.3	99	0.13	1.73	99	0.04
Soluble sugars	0.19	99	0.33	1.90	99	0.09	1.45	99	0.11	1.09	99	0.15
Soluble proteins	0.93	99	0.16	0.94	99	0.11	1.34	99	0.17	0.31	99	0.28
Amino acid	0.16	99	0.35	0.45	99	0.25	0.01	99	0.47	2.63	99	0.04

$P < 0.05$  was bolded, indicating significant differences



**Fig. 3.** RDA (scaling = 2) showing correlation between leaf structural characteristics, biochemicals, and the EPG variables of *Apolygus lucorum*. The angle between lines indicates the correlation between the corresponding variables. Four EPG variables were used, including probing duration per insect (prob dur per ins), mean number of probes per insect (num prob), waveform duration per insect for cell rupture (cell rupture), and waveform duration per insect for ingestion (ingestion).

whereas our study directly analyzed the impact of specific leaf substances on the specific probing behavior to of *A. lucorum*. Our study more deeply and intuitively explained the internal mechanism for greater acceptance of young, tender plants by *A. lucorum* than did other studies. Different substances, or even the same kind of substances, can have different effects on cotton resistance to insects at different growth stages of cotton plants (Lin et al. 2011). For example, wax content of cotton leaves at the bud stage was significantly and positively correlated with cotton plant resistance to *A. lucorum*, whereas wax content of cotton leaves at the seedling stage and the flowering and boll-forming stages had no correlation with plant resistance to *A. lucorum* (Luo et al. 2011b).

The pest *A. lucorum* has many host plants and continuously invades and spreads among different hosts. Clarification of the reasons driving the patterns of spread of *A. lucorum* is the key to developing regional preventive and management technologies for *A. lucorum*. When *A. lucorum* lands on the surface of a plant, it uses its stylets to tentatively probe the food to identify the physical properties of the plant and uses its senses of taste to evaluate the nutrients and resistance chemical substances in the plant to determine whether the plant is suitable for probing. If the plant is not suitable for probing, *A. lucorum* will constantly migrate to different hosts, following the same probing strategy until it finds a suitable plant.

There are three possibilities that might provide sensory stimuli to affect *A. lucorum* host suitability: 1) putative nutrients (sugars, proteins, and amino acids); 2) putative defensive chemical substances (tannins and gossypol); and 3) physical properties of the leaves (age, thickness, and wax content). According to the results of this study, leaf age had the greatest influence on the host selection of *A. lucorum*. This pest might first select young leaves that are easier to probe. After it penetrates the leaf with its stylets, a low content of defensive substances (e.g., gossypol), high content of nutrients (e.g., soluble sugars and amino acids), and a low-soluble protein content improve the stylet probing duration of *A. lucorum*. Thus, our results support the hypothesis that the combination of increased defensive substances, decreased nutrients, and leaf thinness lead to the greater acceptance of young leaves by *A. lucorum*. Our findings will provide further directions to continue to improve host plant resistance to *A. lucorum* in the future.

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## References Cited

- Almeida, R. P. P., and E. A. Backus. 2004. Stylet penetration behaviors of *Graphocephala atropunctata* (Signoret) (Hemiptera, Cicadellidae): EPG waveform characterization and quantification. *Ann. Entomol. Soc. Am.* 97: 838–851.
- Backus, E. A., J. Habibi, F. Yan, and M. Ellersieck. 2005. Stylet penetration by adult homalodisca coagulata on grape: electrical penetration graph waveform characterization, tissue correlation, and possible implications for transmission of *Xylella fastidiosa*. *Ann. Entomol. Soc. Am.* 98: 787–813.
- Backus, E. A., A. R. Cline, M. R. Ellersieck, and M. S. Serrano. 2007. *Lygus hesperus* (Hemiptera: Miridae) Feeding on Cotton: new methods and parameters for analysis of nonsequential electrical penetration graph data. *Ann. Entomol. Soc. Am.* 100: 296–310.
- Backus, E. A., F. A. Cervantes, L. Godfrey, W. Akbar, T. L. Clark, and M. G. Rojas. 2018. Certain applied electrical signals during EPG cause negative effects on stylet probing behaviors by adult *Lygus lineolaris* (Hemiptera: Miridae). *J. Insect Physiol.* 105: 64–75.
- Backus, E. A., F. A. Cervantes, R. N. C. Guedes, A. Y. Li, and A. C. Wayadande. 2019. AC–DC electropenetrography for in-depth studies of feeding and oviposition behaviors. *Ann. Entomol. Soc. Am.* 112: 236–248.
- Cervantes, F. A., E. A. Backus, L. Godfrey, W. Akbar, and T. L. Clark. 2016. Characterization of an EPG waveform library for adult *Lygus lineolaris* and *lygus hesperus* (Hemiptera: Miridae) feeding on cotton squares. *Ann. Entomol. Soc. Am.* 109: 684–697.
- Cervantes, F. A., E. A. Backus, L. Godfrey, C. Wallis, W. Akbar, T. L. Clark, and M. G. Rojas. 2017. Correlation of electropenetrography waveforms from *Lygus lineolaris* (Hemiptera: Miridae) feeding on cotton squares with chemical evidence of inducible tannins. *J. Econ. Entomol.* 110: 2068–2075.
- Chen, J. L. 2001. The biochemical mechanism of wheat resistance to aphids and G protein expression in insect cell lines, purification and activity assays, pp. 12–20. Chinese Academy of Agricultural Sciences, Beijing, China.
- Chen, Z. C., L. Su, F. Ge, and J. W. Su. 2010. Electroantennogram responses of the green leaf bug, *Lygus lucorum* Meyer-Dür (Hemiptera: Miridae), to sex pheromone analogs and plant volatiles. *Acta Entomol. Sin.* 53(1): 47–54.
- Cline, A. R., and E. A. Backus. 2002. Correlations among AC electronic monitoring waveforms, body postures, and stylet penetration behaviors of *Lygus hesperus* (Hemiptera: Miridae). *Environm. Entomol.* 31: 538–549.
- Harrewijn, P., W. F. Tjallingii, and C. Mollema. 1996. Electrical recording of plant penetration by western flower thrips. *Entomol. Exp. Appl.* 79: 345–353.
- Hu, Z., H. Zhao, and T. Thieme. 2013. Modification of non-vector aphid feeding behavior on virus-infected host plant. *J. Insect Sci.* 13: 28.
- Janssen, J. A. M., W. F. Tjallingii, and J. C. van Lenteren. 1989. Electrical recording and ultrastructure of stylet penetration by the greenhouse whitefly. *Entomol. Exp. Appl.* 52: 69–81.
- Johnson, D. D., and G. P. Walker. 1999. Intracellular punctures by the adult whitefly *Bemisia argentifolii* on DC and AC electronic feeding monitors. *Entomol. Exp. Appl.* 92: 257–270.
- Kindt, F., N. N. Joosten, D. Peters, and W. F. Tjallingii. 2003. Characterisation of the feeding behavior of western flower thrips in terms of electrical penetration graph (EPG) waveforms. *J. Insect. Physiol.* 49: 183–191.
- Li, L. M., X. Y. Men, B. H. Ye, Y. Yu, and A. S. Zhang. 2013. Damage of mirid bug *Apolygus lucorum* to winter jujube at different growth stages. *Acta Phytopy. Sin.* 40: 545–550.
- Li, L. M., X. Y. Men, B. H. Ye, Y. Yu, A. S. Zhang, L. L. Li, X. H. Zhou, and Q. Y. Zhuang. 2014. Feeding damage characteristics of *Apolygus lucorum*

- (Hemiptera: Miridae) to different growth stages of cotton. *Acta Entomol. Sin.* 57: 449–459.
- Lin, F. M., D. Wu, Y. H. Lu, Y. J. Zhang, M. Wang, and K. M. Wu. 2011. The relationship between the main secondary metabolites and the resistance of cotton to *Apolygus lucorum*. *Acta Phytopy. Sin.* 37: 165–171.
- Lu, Y. H., Y. Z. Yang, Y. Yin, and Y. S. Yu. 2004. Advances in the resistance and relevant genetic mechanism of cotton to *Aphis gossypii*. *Entomol. Knowl.* 41: 291–294.
- Lu, Y., K. Wu, Y. Jiang, B. Xia, P. Li, H. Feng, K. A. Wyckhuys, and Y. Guo. 2010. Mirid bug outbreaks in multiple crops correlated with wide-scale adoption of Bt cotton in China. *Science* 328: 1151–1154.
- Lu, Z., S. Dong, C. Li, L. Li, Y. Yu, X. Men, and S. Yin. 2020. Sublethal and transgenerational effects of dinotefuran on biological parameters and behavioural traits of the mirid bug *Apolygus lucorum*. *Sci. Rep.* 10: 226.
- Luo, J. Y., J. J. Cui, and Q. Huang. 2011a. The relationship between the content of cotton leaf chlorophyll and waxiness and leaf thickness and the cotton resistance to *Apolygus lucorum*. *Acta Phytopy. Sin.* 8: 320–326.
- Luo, J. Y., J. J. Cui, C. Y. Wang, H. J. Xin, S. Zhang, and L. M. Lv. 2011b. Relationship between the contents of protein, soluble sugar and anthocyanidins in cotton leaf and their resistance to *Apolygus lucorum* Meyer-Dür. *Nat. Sci. Ed.* 39: 75–80, 89.
- Luo, J. Y., J. J. Cui, C. Y. Wang, and H. J. Xin. 2012. Relationship between contents of gossypol acetic acid (GAA) and tannin in cotton leaf and resistance to *Apolygus lucorum* Meyer-Dür. *Cotton Sci.* 24:279–283.
- Men, X. Y., F. Ge, C. A. Edwards, and E. N. Yardim. 2005. The influence of pesticide applications on *Helicoverpa armigera* Hübner and sucking pests in transgenic Bt cotton and non-transgenic cotton in China. *Crop Prot.* 24: 319–324.
- Miao, J., and B. Y. Han. 2008. DC-EPG analysis on effect of tea plant induced by methyl salicylate against feeding of tea green leafhopper. *Acta Phytopy. Sin.* 35: 143–147.
- Nibouche, S., T. Brevault, C. Klassou, D. Dessauw, and B. Hau. 2008. Assessment of the resistance of cotton germplasm (*Gossypium* spp.) to aphids (Homoptera: Aphididae) and leafhoppers (Homoptera: Cicadellidae, Typhlocybinae): methodology and genetic variability. *Plant Breed.* 127: 376–382.
- Pan, H., Lu, Y., Wyckhuys, K. A., & Wu, K. 2013. Preference of a polyphagous mirid bug, *Apolygus lucorum* (Meyer-Dür) for flowering host plants. *PLoS One* 8: e68980.
- Song, H. Y., Z. B. Lu, L. L. Li, Y. Yu, A. S. Zhang, Q. Y. Zhuang, X. H. Zhou, Y. F. Zhai, H. Chen, and X. Y. Men. 2017. EPG analysis of *Apolygus lucorum* Meyer-Dür feeding behaviors on cotton leaves at different growth stages. *China Cotton* 44: 15–19.
- Tang, D. L., and W. G. Wang. 1996. Effect of induced-resistance of cotton on the development and behaviour of cotton bollworm *Helicoverpa armigera* (Hübner). *Acta Gossypii Sin.* 8:276–278.
- Tuelher, E. S., E. A. Backus, F. Cervantes, and E. E. Oliveira. 2020. Quantifying *Lygus lineolaris* stylet probing behavior and associated damage to cotton leaf terminals. *J. Pest Sci.* 93: 663–677.
- Xu, R. M., Y. Zhang, and W. R. Ma. 1994. The probing and feeding process of the greenhouse whitefly, *Trialeurodes Vaporariorum* Westwood. *Entomol. Sin.* 1: 67–76.
- Yang, Y. H., Q. W. Zhang, and X. X. Liu. 2013. The relationship between the contents of nutrients and tannins in different cotton varieties and their resistance to *Lygus lucorum*. *Scie. Agric. Sin.* 46: 4688–4697.
- Young, N. Y. 1998. Electrically recorded feeding behavior of *Nephotettix cincticeps*. *J. Asia-Pac. Entomol.* 1: 147–161.
- Zhang, W. Z., J. Li, X. L. Zhou, Q. L. Li, Z. L. Hu, L. L. Zhao, Z. H. Liu, and F. Han. 2018. Research progress on *Apolygus lucorum* (Meyer-Dür). *J. Agric.* 8: 13–18.
- Zhao, M., J. C. Ji, X. R. Guo, and F. M. Yan. 2011a. Advance in the studies on the resistance mechanism of piercing-sucking insects on host plants. *Insect Res. Central China* 7: 163–167.
- Zhao, Q. J., D. Wu, F. M. Lin, C. Y. Li, Y. J. Zhang, K. M. Wu, and Y. Y. Guo. 2011b. EPG analysis of *Apolygus lucorum* Meyer-Dür feeding behaviors on different cotton varieties (lines) and field verifications. *Sci. Agric. Sin.* 44: 2260–2268.