## USE OF VIDEO ASSAYS TO ASSESS FEEDING BEHAVIOR BY LYGUS HESPERUS KNIGHT (HEMIPTERA: MIRIDAE) W. Rodney Cooper Dale W. Spurgeon USDA-ARS-WICSRU Shafter, CA

### <u>Abstract</u>

Lygus hesperus Knight (Hemiptera: Miridae) is a key cotton pest in the western United States that injures and induces abscission of squares and small bolls. Feeding behavior varies among individual lygus, and this variation complicates interpretation of studies to elucidate lygus/cotton interactions. We developed video-based methods to monitor lygus activities and investigate factors that influence feeding behavior. We compared times allocated to feeding and trivial movement between male and female adult lygus of three different reproductive states: prereproductive, reproductive and mated, and reproductive and unmated. Pre-reproductive adults spent more time probing/feeding compared to reproductive adults, but there were no differences between genders of pre-reproductive adults. Behaviors of reproductive adults were influenced by both gender and mating status; the effects of mating status on trivial movements and time spent probing were roughly opposite for the two genders. Unmated reproductive females exhibited less trivial movement and fed with longer probing intervals compared to mated females. Conversely, unmated males exhibited more trivial movement and spent less time feeding compared to mated males. Mated females also tended to exhibit more trivial movement than mated males, but their durations of probing were similar. In contrast, reproductive unmated females exhibited less trivial movement and spent more time probing than unmated males. This initial study documents previously unrecognized sources of variation in adult lygus behavior that may prove useful in developing a better understanding of variation in square loss induced by adult lygus.

#### **Introduction**

Damage to cotton caused by *Lygus hesperus* Knight can be highly variable, and observed fruit losses are often inconsistent with estimates of lygus populations (Rosenheim et al., 2006). These inconsistencies complicate management decisions and hamper efforts to unambiguously elucidate lygus/cotton interactions (Scales and Furr, 1968; Gutierrez et al., 1977; Leigh et al., 1988; Mauney and Henneberry, 1984). Sources of variability proposed to explain the inconsistencies in lygus-induced damage include behavioral differences among lygus stages, and variability in the hosts' responses to lygus feeding (Rosenheim et al., 2006). Previous studies that investigated lygus stage–dependent damage to cotton have resulted in conflicting conclusions. Some studies indicate that adults cause more damage than nymphs (Gutierrez et al., 1977; Zink and Rosenheim, 2005), while other studies indicate the opposite (Jubb and Carruth, 1971) or indicate no differences between damage caused by nymphs and adults (Rosenheim et al. 2006). Because of the inconsistencies among published studies, it is not possible to explicate clear patterns corresponding to lygus stage-dependent square losses.

Although previous studies have compared plant injury caused by different lygus genders and life-stages, few studies have attempted to describe or compare gender or age-dependent feeding behaviors (but see Zink and Rosenheim, 2005). An improved understanding of lygus behavior will allow us to standardize experiments and relate square losses to variations among lygus stage-dependent behaviors. In the current study, we use video-based methods to monitor lygus trivial movement and feeding behaviors, and to compare these behaviors between genders and among adults of different reproductive states (pre-reproductive, reproductive and mated, and reproductive and unmated).

### **Materials and Methods**

### Arena Setup

Cotton squares (6-7 mm diameter) were collected prior to each assay from a field planting of Acala cotton (PhytoGen 72) located at the Shafter Research and Extension Center in Shafter, CA. Squares were placed in a plastic bag with a moist paper towel, and kept cool during transport to the lab. Bracts were removed from each square prior to assays to facilitate unimpeded view of lygus behaviors. Each square was rinsed with deionized water and partially embedded horizontally in paraffin wax within a 50-mm petri dish lid (Fisher Scientific, Pittsburgh, PA). Squares were positioned to expose half of the square to lygus feeding, to provide unobstructed views of

feeding events. Since the absence of extra-floral nectaries inhibit the attraction of lygus to cotton (Meredith et al. 1973), at least one nectary at the base of each square was left exposed. After the wax was thoroughly cooled, a single lygus adult was placed on the surface of the hardened wax and confined to the arena with the petri dish bottom.

We compared behaviors among lygus gender and reproductive states (pre-reproductive, reproductive and mated, and reproductive and unmated) (n=5/gender/state; N=30). Lygus adults typically mate 3-5 days after adult eclosion (Strong et al. 1970; DWS and WRC personal observations), thus adults <3 days old were considered pre-reproductive. Most lygus adults kept in mixed colonies are mated by 7 days after adult eclosion (Strong et al. 1970; DWS and WRC personal observations), thus 7-10 d old adults were considered reproductive. Newly eclosed lygus adults were pulled from a laboratory colony maintained on green beans (*Phaseolus vulgaris* L.) and sunflower seeds (*Helianthus annuus* L.). Pre-reproductive adults obtained on the day of eclosion were held individually in 5-dram plastic vials within a growth chamber (25°C, 14L: 10D) for 1-3 days prior to assays. Each vial was closed with a ventilated lid and contained a green bean segment with ends sealed with paraffin wax. Unmated reproductive lygus were held individually for 7-10 days prior to assays as described for pre-reproductive adults. Mated reproductive lygus were held in mixed-gender groups of six (3/gender) for 7-10 days in clear plastic rearing containers (15 cm diameter, 8 cm height) containing green beans. Mated females were dissected following video assays to confirm that they were mated based on the presence of a spermatophore.

### Lygus Behaviors

Each lygus was recorded individually for 6 hrs using a Canon Visualizer RE-450X (Canon U.S.A. Inc., Lake Success, NY) and Windows Movie Maker 2.1 (Microsoft Corporation, Redmond, WA) at a rate of 20 frames per second. Overhead lights of the Canon Visualizer were supplemented with a small fluorescent lamp. Arenas, lights, and the camera were covered with a white cloth to minimize disturbance to the lygus by other activities in the laboratory.

Probing events were monitored by directly viewing stylet probing, which begins when the proboscis bends between the 3<sup>rd</sup> and 4<sup>th</sup> segments. When views of the proboscis were obstructed, antennal and body postures were used to monitor feeding behaviors as described by Backus et al. (2007). Time points when lygus moved on or off squares (referred to as trivial movement) and start and stop times of each probing event (referred to as feeding behaviors) were recorded.

# **Statistical Analyses**

Dependent variables for analyses of trivial movements were 1) the average time interval on the square, 2) average time interval off the square, 3) number of times leaving the square and 4) total time on the square. Dependent variables for analyses of feeding behaviors were 1) number of probing events 2), total time probing, 3) average probing interval, 4) the proportion of time spent probing while the bug was on the square and 5) number extra-floral nectary probes. Data were examined for heterogeneity of variance and non-normality of errors by inspecting residual and normal quantile-quantile plots, respectively. Based on these plots, proportional data (time probing/time on square) were arcsine square-root transformed and all other data were natural-log transformed prior to analyses. Untransformed means and standard errors are presented. All analyses were conducted using PROC GLIMMIX (SAS ver. 9.2, SAS Institute, Cary, NC) with gender, reproductive state, and their interaction as independent variables. Each assay, which consisted of a different lygus adult and a fresh square, was considered the experimental unit.

# **Results and Discussion**

# Lygus Trivial Movement

There was a significant interaction between gender and reproductive status for the total time on squares (Table 1A). Unmated reproductive males spent less total time on the squares compared to other combinations of gender and reproductive states (46% vs an average of 87%, respectively; Table 2A). There was also a significant interaction between gender and reproductive status for the average time interval on squares (Table 1A). Unmated reproductive males exhibited the shortest average time interval on squares compared to other combinations of gender and reproductive state (Table 1B). Additionally, mated females exhibited a shorter average time interval on squares than other combinations of gender and reproductive state except unmated males. Finally, there was a significant interaction between gender and reproductive status for the number of times lygus left the squares (Table 1A). Mated

reproductive females and unmated reproductive males left the squares more frequently  $(>4\times)$  than any other combination of gender and reproductive state (Table 2A).

The average time interval when bugs were off the squares was influenced by lygus gender, but not by reproductive status (Table 1A). Absence of a significant interaction indicated that gender effect was exhibited for all reproductive states (Table 1A). The mean time interval lygus spent off the squares was  $4 \times \text{longer}$  for males ( $10.0 \pm 2.4 \text{ min}$ ) compared to females ( $2.7 \pm 0.7 \text{ min}$ ).

Table 1. Factorial analysis of lygus trivial movement (A) and feeding behaviors (B) on 6-7-mm diameter cotton squares with gender, reproductive status, and their interactions as independent variables.

		Gender	Reproductive status	Interaction
Variable		$F_{1, 24}(P)$	$F_{2, 24}(P)$	$F_{2, 24}(P)$
А	Total time on square	10.5 (<0.01)	3.9 (0.04)	5.1 (0.01)
	Avg. time interval on square	2.9 (0.10)	3.5 (0.05)	8.4 (<0.01)
	No. times leaving square	0.8 (0.39)	3.4 (0.05)	7.9 (<0.01)
	Avg. time off square	12.9 (<0.01)	0.48 (0.63)	0.05 (0.95)
В	No. probing events	4.1 (<0.06)	4.2 (0.03)	3.4 (0.05)
	Total probing time	5.0 (0.04)	6.5 (<0.01)	2.1 (0.15)
	Min probing interval	1.9 (0.18)	1.6 (0.22)	0.6 (0.55)
	Max probing interval	2.7 (0.11)	2.6 (0.09)	6.3 (<0.01)
	Avg. probing interval	0.2 (0.65)	9.9 (<0.01)	3.6 (0.05)
	Proportion of time probing while	1.8 (0.20)	4.8 (0.02)	0.27 (0.76)
	on square			
	No. extrafloral nectary probes	0.37 (0.55)	3.1 (0.06)	1.3 (0.30)

Table 2. Influence of lygus gender and reproductive status on lygus trivial movement	t and feeding behaviors on 6-7-
mm diameter cotton squares. Lygus were exposed to cotton squares for 360 min. V	Values for time are presented in
minutes. Means within a column followed by the same letter are not significantly dif	ferent ( $\alpha$ =0.05).

Gender	Reproductive	Trivial Movement Behaviors		Feeding Behaviors		
		Total time on square	Avg. time interval on square	No. times leaving square	No. probing events	Avg. probing interval
Female	Pre- reproductive	319.3±10.8a	126.6±57.1a	3.4±1.3b	17.6±3.8b	14.0±2.9a
	Reproductive Mated	318.5±19.8a	31.3±11.3b	22.4±11.9a	57.6±9.5a	3.1±1.1c
	Reproductive Unmated	347.1±50.8a	97.2±32.5a	4.8±1.9b	21.6±2.9b	8.0±4.0ab
Male	Pre- reproductive	284.0±35.7a	53.1±9.1ab	4.6±0.8b	15.8±32b	14.5±4.5a
	Reproductive Mated	303.0±6.8a	71.3±20.5ab	4.4±1.1b	21.4±2.2b	5.4±1.2bc
	Reproductive Unmated	164.7±30.7b	12.2±5.0c	21.8±6.9a	19.0±4.9b	2.3±0.6c

Our observations indicate that gender has little influence on the extent of trivial movement of pre-reproductive ( $\leq$ 3 days old) adults. In contrast, movement behavior of reproductive adults (7-10 days old) was influenced by both gender and mating status. Unmated reproductive males spent less total time on squares, stayed on squares for the shortest intervals, and left squares more frequently than did pre-reproductive or mated reproductive males. In contrast, mated females appeared more active (left the square more often and for longer intervals) than unmated reproductive females. Thus, previous mating exerted an opposite effect on trivial activity of respective genders of reproductive lygus. These results suggest a pattern in which pre-reproductive adults are relatively inactive, whereas the activity levels of reproductive adults are generally greater but also depend on whether they had previously mated. It is possible the opposite influences of mating on the respective activity patterns of reproductive males and females may be related to the different requirements imposed by mate seeking, mate attraction, and oviposition. For

example, unmated males may exhibit high levels of activity associated with searching for unmated females, whereas unmated females may be capable of attracting mates from a single location. After mating, male activity may diminish because of the need to replenish contents of the accessory glands, whereas female activity may increase out of need to locate oviposition sites. In this scenario, activity of recently mated males might be expected to increase once contents of the accessory glands have been replenished, and this hypothesis can be tested.

## **Lygus Feeding Behaviors**

There were significant interactions between gender and reproductive status in the total number of stylet probes (probing events; Table 1B). On average, mated reproductive females probed about 3 times more often than other combinations of gender and reproductive state. Both gender and reproductive status influenced the total probing time (Table 1B), and no interaction between these effects was observed (Table 1B). Overall, total probing time for females (167.1  $\pm$  20.3 min) was greater than for males (109.1  $\pm$  18.7 min), and total probing time for prereproductive adults (191.9  $\pm$  15.4 min) was greater than for mated (131.5  $\pm$  22.2 min) or unmated adults (91.1  $\pm$  27.4 min). There was a significant gender by reproductive-status interaction for average probing times (Table 1B). The mean probing intervals of pre-reproductive males and females were of longer duration than those of mated reproductive females and unmated reproductive males (Table 2B). Probing intervals of unmated females and mated males were intermediate in duration (Table 2B). Reproductive status affected the proportion of time spent probing while on squares, and absence of a gender by reproductive status interaction suggested the effects of reproductive status were similar for both bug genders (Table 1B). While on squares, pre-reproductive adults devoted a larger proportion of time to probing  $(0.63 \pm 0.03)$  than did mated  $(0.42 \pm 0.07)$  or unmated reproductive adults  $(0.36 \pm 0.03)$ 0.08). The mean number of times lygus adults probed extrafloral nectaries did not differ between lygus genders, among reproductive classes, or among combinations of gender and reproductive status (Table 1B). However, there was a numerical trend for higher numbers of probes in the nectaries by pre-reproductive bugs  $(2.2 \pm 0.5)$  than by mated  $(1.4 \pm 0.4)$  or unmated reproductive bugs  $(0.7 \pm 0.2)$ .

Patterns observed for feeding behavior were generally the opposite of those observed for trivial movement. Prereproductive adults spent more total time feeding/probing, and stylet-probed for longer intervals compared to reproductive adults. Also, gender did not influence feeding behaviors of non-reproductive adults. It is possible that both genders of pre-reproductive adults require relatively long feeding bouts to build energy reserves in preparation for mating and oviposition behaviors. In contrast, feeding behaviors of reproductive adults were influenced by both gender and mated status. Unmated males probed as many times as mated males, but probed in shorter time intervals. Also, mated females probed >2.5 times as often, but with shorter probes, than unmated reproductive females. Mated females also exhibited oviposition behaviors, which may explain the large number of short interval stylet-probes. Prior to each oviposition attempt, regardless of whether an egg was actually laid, mated females stylet-probed the oviposition site  $\approx 1$  second prior to extending the ovipositor toward the substrate. Potentially, many of the short stylet-probes observed for mated females were associated with searching behaviors for suitable oviposition locations (Romani et al., 2005). Saliva injected by lygus while feeding causes injury to cotton (Strong and Kruitwagen 1968), but it is unknown whether females inject saliva during stylet-probes associated with ovipositional behaviors, or if these stylet-probes are entirely chemo-receptive. If saliva is injected during oviposition stylet-probes, then mated females could potentially cause more damage to cotton than other combinations of gender and reproductive state. The roles of stylet-probes prior to oviposition, beyond searching behaviors, and their potential consequences to plant injury, require further investigation. Regardless, the combined observations of male and female probing behaviors yielded patterns that were consistent with our hypothesis to explain variations in movement behaviors; that differences in observed feeding behaviors among male and female reproductive lygus may be driven by different priorities (mate searching, mate attraction, oviposition) of these respective groups.

### **Summary**

Our study indicates that trivial movement and feeding behaviors differ little between pre-reproductive male and female adults ( $\leq$ 3 days old). However, once adults reach a reproductive age (>7 days after eclosion), behaviors tend to differ between males and females, and are further influenced by mating history. In general, unmated reproductive females spent more time on squares and feeding compared to mated females. After mating, females spent more time away from square and fed in shorter intervals, but stylet-probed squares more often compared to unmated females. Patterns in male behaviors were opposite of those of females. Unmated males left squares more often and fed less than mated males. After mating, males spent more time on squares and feeding compared to unmated males. It is

currently unknown how these behaviors may influence square-loss rates, but it is clear that comparisons of square losses induced by different genders of lygus adults are incomplete in the absence of information regarding adult age and mating status. Furthermore, general comparisons of square losses caused by lygus adults and nymphs may be severely influenced by the vast behavioral variation among lygus adults.

Although previous studies have compared injury responses of cotton caused by lygus adults and nymphs, or adult males and females, the results of these studies have varied (Jubb & Carruth, 1971; Gutierrez et al., 1977; Zink & Rosenheim, 2005; Rosenheim et al., 2006). No previous study has compared injury responses among combinations of adult lygus gender and reproductive status. Our results suggest that studies to investigate lygus/cotton interactions and lygus-induced square loss could be improved by accounting for sources of variation in lygus feeding behavior. We also demonstrate that video-based behavioral studies can be useful in identifying such sources of variation. We do not conclude, on the basis of available data, that movement and feeding times determined from video monitoring are representative of those occurring in the field. However, the variations we observed in behavioral responses among combinations of bug gender and reproductive status may correlate to variations in feeding injury caused by different classes of adults. These correlations can be further examined in greenhouse or field studies, and if proven, would provide information critical to the design of improved studies of lygus/cotton interactions.

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## **Disclaimer**

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