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ARTHROPOD MANAGEMENT

Accuracy of *Lygus hesperus* Knight (Hemiptera: Miridae) Egg Counts Improves with Egg Development

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ABSTRACT

The western tarnished plant bug, *Lygus hesperus* Knight, is a key cotton *(Gossypium* spp.) pest, managed primarily by application of insecticides according to nominal thresholds. Efforts to reduce reliance on insecticide-based tactics will require a more astute understanding of the physiological ecology of *L. hesperus* than is currently available. A key biological parameter that may be manipulated through cultural or genetic means is reproduction. Estimates of *L. hesperus* oviposition are commonly obtained by visual inspection, but the accuracy of those estimates has been questioned. Because the eggs swell during development, we hypothesized that delaying counts of eggs to permit some development might improve sampling accuracy. Estimates of *L. hesperus* egg numbers were obtained immediately after oviposition and again at three days after oviposition, and counts at both times were regressed with total eggs determined by the sum of either hatched eggs or the number of nymphs, and unhatched eggs. Sampler experience was a major factor influencing fidelity of egg counts with total eggs, but a single repetition of the experiment accompanied by knowledge of the results was sufficient to optimize sampler effectiveness. Regressions relating total eggs to counts, whether the counts were taken immediately after oviposition or after three days, were significant. However, compared with counts of newly-laid eggs, the regression relating egg counts at three days with total eggs was more stable and showed better agreement. These results can be used to maximize statistical power and minimize sampling errors in future examinations of *L. hesperus* oviposition.

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Improved management and availability of reduced-risk insecticides have diminished the economic importance of lygus bugs in some western cotton (*Gossypium* spp.) production regions (Anonymous, 2013). Despite these advances, lygus bugs (primarily *Lygus hesperus* Knight) remain the most important pest complex in western cotton (Williams, 2016). However, as pest management improves and economic impact declines, additional improvement becomes more difficult using the traditional model of pest management. For this reason, the Pest Management and Biocontrol Research Unit, Maricopa, AZ, is focused on developing ecologically-based management tactics that maximize environmental or genetic-based resistance to reduce lygus reproduction, survival, or crop injury. This approach will require improved understanding of the molecular biology of *L. hesperus*, and more detailed knowledge of its basic biology and physiological ecology.

Detailed knowledge of the impacts on *L. hesperus* reproduction of environmental stressors, such as those induced by high temperatures typical of the arid and semi-arid production regions of the West, is lacking. Design of experiments with sufficient statistical power to discern subtle, but ecologically important responses to environmental conditions would be enhanced if accuracy of lygus egg counts could be maximized. Many species of mirid, including *Lygus* spp., insert their eggs within host tissue and often only the operculum is visible (Stewart and Gaylor, 1993; Wheeler 2001). In some studies of *Lygus* and similar species, the detection of eggs was presumably enhanced by staining with ethanolic Safranin O (Alvarado-Rodriquez et al., 1986; Benedict et al., 1981; Ferran et al., 1996). However, this method would likely influence egg survival if subsequent observations are needed of the same cohort of individuals. In at least one study of *L. hesperus* parasitism, eggs were counted after hatching (Jackson, 2003). In most studies of *Lygus* spp. oviposition, eggs in a wide variety of host plants have been detected by visual observation, often

with the aid of a dissecting microscope (Abel et al., 2010; Balachandran et al., 2014; Brent et al., 2011; Conti et al., 2012; Elmore, 1955; Noma and Strickler, 2000; Ugine, 2011, 2012). During studies of *L. hesperus* egg development (Cooper and Spurgeon, 2012, 2013), it was observed that, despite careful examination, the numbers of eggs detected visually in pods of green bean (*Phaseolus vulgaris* L.) were often lower than the numbers of resultant nymphs (W.R.C. and D.W.S., unpublished data). This observation raises questions regarding the accuracy of visual egg counts of *Lygus* spp.

Cooper and Spurgeon (2012) reported that fertilized eggs of *L. hesperus* swell during the early stages of development. Steward and Gaylor (1993) reported similar observations for *L. lineolaris*. It seems plausible that after swelling, partially developed eggs may be more apparent and more accurately sampled than are newly deposited eggs. Our objectives were to estimate the relationships between total *L. hesperus* eggs and counts of newly hatched and three-d-old eggs, respectively, and to examine the effects of sampler experience on these relationships.

MATERIALS AND METHODS

The experiment used gravid adult female *L. hesperus* originating from fields of alfalfa (*Medicago sativa* L.) near Maricopa, AZ, or the F_2 progeny of the field-collected females. The field-collected females were used in four experimental repetitions, and their F_2 progeny were used in two repetitions. Prior to the experiments the insects were maintained in the laboratory on pods of green bean and raw seeds of sunflower (*Helianthus annuus* L.) at 27±2°C with a 14:11 (L:D) h photoperiod as described by Spurgeon and Brent (2015).

In each repetition of the experiment, each female *L. hesperus* was held individually within a 150 × 15 mm Petri dish. A 150-mm diameter disk of Whatman No. 2 filter paper was centered in the Petri dish lid and the perimeter of the disk was pleated so it fit the lid tightly. When the Petri dish bottom was in place the filter paper inhibited the escape of hatching nymphs. Each female was provided an intact green bean pod for feeding and oviposition. The Petri dishes with females were held on the laboratory counter (~26–28°C) under fluorescent lighting for six–eight h before the females were removed and the initial count of eggs was conducted. Eggs were counted with the aid of a dissecting microscope, and each egg was identified by a number on the pod using black permanent ink (Pigma Micron 005, Sakura Color Products, Osaka, Japan). Marking of the eggs facilitated later examinations for hatching, and minimized the potential to count the same egg more than once.

Each sampler was randomly assigned five bean pods for egg counts. After recording the initial (zero-d) egg counts, each bean was returned to its dish which was wrapped in Parafilm (Pachiney Plastic Packaging, Chicago, IL) to prevent desiccation of the developing eggs. Dishes with eggs were held in an environmental chamber maintained at 26.7±0.5°C with a 14:10 (L:D) h photoperiod. When the eggs were three days old, the beans were reexamined by the same samplers and any previously undiscovered eggs were numbered and recorded. Sampling times were not recorded but were generally less than one h to sample five beans. The longest sampling times were associated with higher egg counts primarily because of the time necessary to number eggs.

After the three-d sampling, dishes were again wrapped in Parafilm and returned to the environmental chamber. Dishes were examined daily for excess condensation during the egg development period. When condensation was evident, the dishes were opened, the condensate was removed by blotting, and the dishes were resealed with Parafilm. Removal of condensate reduced the potential for data loss from mold growth. Beginning on the fifth day after oviposition, the Petri dishes were examined at least twice daily for hatching nymphs. At each inspection, newly-hatched nymphs were tallied and removed to reduce the potential for egg predation by the nymphs. Inspections of the dishes continued until no additional hatch was observed, typically through the eighth day after oviposition. At the completion of hatching, each bean was inspected for marked or unmarked eggs that remained unhatched. The numbers of hatched and unhatched eggs were recorded to permit estimation of hatch rates. Egg hatch was identified by an empty chorion and exertion of the serosal cuticle (Cooper and Spurgeon, 2012). Eggs that were collapsed or desiccated were typically amber in color and were recorded as dead. Total eggs were estimated by adding the numbers of nymphs to the numbers of unhatched eggs, except on the few occasions when the number of hatched eggs was higher than the number of nymphs detected. In this latter case, total eggs equaled the sum of hatched and unhatched eggs.

Three samplers participated in each of six repetitions of the experiment, except for the fifth repetition

when only two samplers were available. Samplers ranged from no prior experience in sampling *Lygus* spp. eggs to extensive experience. During the week before the first experimental repetition, samplers met to examine beans with eggs and to share observations regarding egg sampling. After each repetition of the experiment, each sampler was informed of the closeness of their counts to total eggs with the anticipation that such feedback might increase proficiency of egg sampling with increased experience. Although we had not initially conceived that some counts would be higher than the numbers of eggs actually present, when counts at three days after oviposition revealed such mistakes those errors were included in the three-d counts. By perpetuating those errors, we assumed the three-day counts more closely approximated independent counts than they would if errors from initial counts were corrected.

All analyses were conducted using SAS (SAS Institute, 2012). Estimates of egg hatch were of interest in the event there was an obvious relationship between egg survival and sampler proficiency. The estimated probability of egg hatch was examined by a generalized linear model (PROC GLIMMIX) with a fixed effect of experimental repetition. A binomial distribution and Laplace estimation were used in a conditional analysis. Pairwise comparisons of the respective probabilities of egg hatch among repetitions were corrected for multiplicity using the ADJUST=SIMULATE option of the LSMEANS statement.

Respective relationships between egg counts immediately after oviposition (zero-d) and at three days after oviposition to total eggs were evaluated separately by mixed-model linear regression using PROC GLIMMIX. In each case, the model contained a random term for sampler so inferences could be extended to reflect expectations for other samplers. Each model was initially evaluated by ANCOVA for a common slope among repetitions of the experiment. When analyses indicated different slopes among repetitions, predicted responses for each repetition were estimated at three points; total eggs = 5, 10 and 20 (Littell et al., 2006). Pairwise comparisons of these predictions among experimental repetitions were then used to distinguish groupings of repetitions with common slopes. The ADJUST=SIMULATE option was used to control experiment-wise type-I error for these multiple comparisons. Each time the data for a repetition was removed from the larger group of data, the ANCOVA process was repeated. All regression analyses used Kenward-Roger corrected degrees of freedom (the DDFM=KR option of the MODEL statement). Changes in the slopes of respective repetitions were interpreted to reflect increased sampler proficiency resulting from experience and feedback.

Concordance (proportion of counts agreeing) of zero-d and three-d egg counts with total eggs and corresponding asymptotic 95% Agresti-Coull confidence intervals were calculated for pooled repetitions two – six using PROC FREQ. A comparison of concordance between zero- and three-d egg counts was made using the Mantel-Haenszel row mean score statistic in contingency tables stratified by repetition of the experiment. In that analysis, the strata are analogous to a blocking variable in an analysis of variance.

RESULTS AND DISCUSSION

Although the majority of marked eggs hatched in all repetitions of the experiment, the estimated probability of hatch differed among repetitions ($F = 4.80$; df = 5, 66; $P < 0.01$). The probability of egg hatch in the first experimental repetition (± S.E., 0.71 ± 0.06) was not different from hatch in the second repetition (0.84 ± 0.04; adjusted-$P = 0.45$) but was lower than for later repetitions (repetition 3, 0.95 ± 0.03; repetition 4, 0.90 ± 0.03; repetition 5, 0.91 ± 0.03; repetition 6, 0.95 ± 0.02; $<0.01 \leq$ adjusted-$P \leq 0.03$). No differences in the probability of egg hatch were indicated among repetitions two – six ($0.23 \leq$ adjusted-$P \leq 1.00$).

Relationship of 0-d Egg Counts to Total Eggs. Tests of common slopes among regressions of egg counts taken on the day of oviposition and total eggs indicated a common intercept among the repetitions ($F = 0.90$; df = 5, 20.5; $P = 0.50$) but heterogeneous slopes ($F = 5.15$; df = 5, 5.22; $P = 0.04$). Estimation of regression parameters by the heterogeneous slopes model suggested that only the slope for the first repetition was not significantly different from zero (repetition one slope and S.E., 0.095 ± 0.137, $t = 0.69$, df = 22.7, $P = 0.50$; repetitions two – six slope, 0.815 – 0.962, $P < 0.01$). Multiple comparisons among predicted values from the respective regressions indicated significant differences between repetition one and repetitions two – five at eggs = 5 and eggs = 20, and differences between repetition one and repetitions two – six at eggs = 10. Adjusted *P*-values comparing slopes from repetitions one and six at eggs = 5 ($P = 0.06$) and at eggs = 20 ($P = 0.05$) were suggestive of heterogeneous slopes. Analyses of the remaining repetitions of the

experiment (two – six) suggested a common intercept ($F = 0.58$; df = 4, 11.1; $P = 0.68$) and slope ($F = 0.12$; df = 4, 5.42; $P = 0.97$) were appropriate. Comparisons of respective predicted values at total eggs = 5, 10, and 20 for repetitions two – six using the unequal slopes model confirmed the equivalence of these estimates ($0.36 \leq$ adjusted-$P \leq 1.00$). Based on these analyses, two regression relationships were calculated; one for the first repetition of the experiment, and one using a common-slopes model for combined repetitions two – six.

The regression for repetition one yielded estimated intercept and slope (± S.E.) of 0.478 ± 0.814 and 0.126 ± 0.167, respectively (Fig 1a). However, the regression was not significant ($F = 0.57$; df = 1, 9.02; $P = 0.47$) and the confidence intervals calculated at total eggs = 5, 10, and 20 all included zero (Table 1). In contrast, the regression relating initial counts to total eggs for repetitions two – six was highly significant ($F = 472.03$; df = 1, 67.7; $P < 0.01$) and yielded an estimated intercept and slope of -0.860 ± 0.301 and 0.936 ± 0.043, respectively (Fig. 1c). Point estimates from this regression at total eggs = 5, 10, and 20 were all different from zero, but the accuracy (percentage of total eggs detected) tended to increase with increasing numbers of eggs (Table 1).

Relationship of Three-d Egg Counts to Total Eggs. Analyses of egg counts taken at three days after oviposition were similar to those taken immediately after oviposition except that the numbers of eggs detected by the samplers were generally increased. The test of a common regression among experimental repetitions again indicated a common intercept ($F = 0.37$; df = 5, 72.2; $P = 0.87$) but heterogeneous slopes ($F = 5.61$; df = 5, 72.1; $P < 0.01$). Model predictions at total eggs = 5, 10, and 20 using the heterogeneous slopes model indicated all of the experimental repetitions yielded estimates that were different from zero ($P < 0.01$). However, pairwise comparisons showed that the estimates from the first repetition were different from the estimates from other repetitions (<0.01 < adjusted-$P < 0.02$). Tests of a common slope among the remaining repetitions (two – six) indicated that a common intercept ($F = 0.47$; df = 4, 59; $P = 0.76$) and slope ($F = 0.70$; df = 4, 59.1; $P = 0.60$) were appropriate. Comparisons of the model predictions at total eggs = 5, 10, and 20 also failed to detect differences among these experimental repetitions ($0.24 <$ adjusted-$P \leq 1.00$). As for the egg counts taken immediately after oviposition, separate regressions relating counts taken three days after oviposition to total eggs were estimated for the first experimental repetition and combined repetitions two – six.

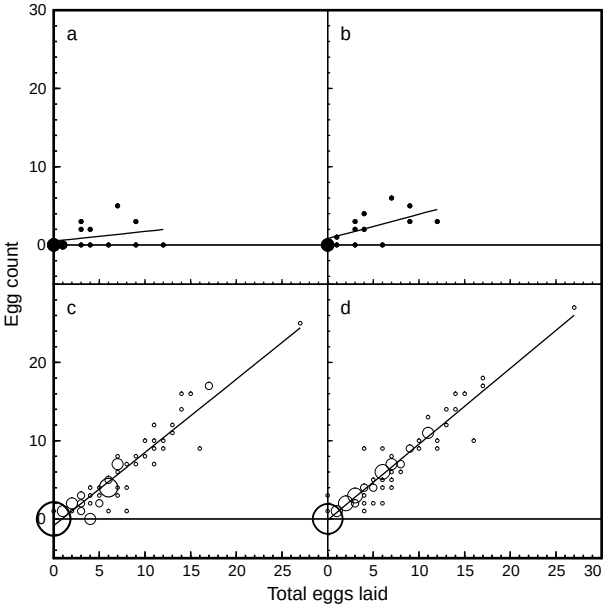

Fig. 1. Scatter plots and regression lines relating counts of *L. hesperus* eggs to total eggs for the first experimental repetition at days 0 (a) or 3 (b) after oviposition, and for combined repetitions 2 – 6 at days 0 (c) or 3 (d) after oviposition. Sizes of the circles are proportional to the number of points represented in each row of graphs.

Table 1. Point estimates and 95% confidence intervals (C.I.) of *L. hesperus* egg numbers from regressions relating counts taken immediately after oviposition to total eggs for an initial (1) and subsequent (2 – 6) experimental repetitions

Experimental repetition[z]	Total eggs	Model-estimated eggs (± S.E.)	95% C.I.	Estimated % of total eggs
1	5	1.11 (± 0.46)	-4.50 — 6.72	22.2
	10	1.74 (± 1.07)	-1.24 — 4.72	17.4
	20	3.00 (± 2.68)	-3.21 — 9.21	15.0
2 – 6	5	3.82 (± 0.14)	2.83 — 4.81	76.4
	10	8.50 (± 0.21)	7.93 — 9.07	85.0
	20	17.86 (± 0.61)	16.64 — 19.08	89.3

[z] Groupings of experimental repetitions were based on tests of common slopes using ANCOVA.

Reasonable estimates of model degrees of freedom for the regression relating egg counts at three days after oviposition to total eggs for the first experimental repetition were not obtained by maximum likelihood so Type III tests were used. The regression estimated the intercept (±S.E.) as 0.814 ± 0.310 and the slope as 0.271 ± 0.066, and the regression was significant (F = 16.96; df = 1, 11; P < 0.01; Fig. 1b). Regardless of the significant relationship, accuracy of these counts was poor and only 31 – 43% of total eggs were detected (Table 2). The regression relating egg counts at three days to total eggs for repetitions two – six was also significant (F = 767.56; df = 1, 66.5; P < 0.01; intercept, -0.149 ± 0.385; slope, 0.970 ± 0.035; Fig. 1d). In contrast with the counts from the first experimental repetition, point estimates from repetitions two – six accounted for ≥94% of total eggs (Table 2).

Concordance of Counts with Total Eggs. In the first experimental repetition, few of the initial egg counts exactly matched the final estimates of total eggs. The proportion of zero-day counts that was concordant with total eggs in the first repetition was 0.267 (C.I. 0.105 – 0.524) and this proportion was unchanged in the egg counts at three days after oviposition. Concordance between zero-day counts and total eggs in repetitions two – six was higher (0.357, C.I. 0.255 – 0.474) and was further increased for the egg counts taken three days after oviposition (0.543, C.I. 0.427 – 0.654). The row mean score test comparing the frequencies of concordant and discordant counts for zero- and three-day egg counts, stratified over repetitions two – six of the experiment, indicated significantly greater concordance for the three-d compared with the zero-d counts (Q_{SMH} = 8.96, df = 1, P < 0.01). These results were consistent with the inferences from the fitted regressions that suggested greater accuracy of egg counts at three days after oviposition compared with counts taken immediately after oviposition.

CONCLUSIONS

Although the first repetition of the experiment differed from most other repetitions in egg hatch and the effectiveness with which samplers identified *L. hesperus* eggs, there was no apparent mechanism by which reduced egg hatch could explain the poor results of these earliest samples. Rather, samplers' knowledge of the inadequacies of those earliest counts at both sampling times prompted refinement of the sampling technique. Some of this improvement resulted from knowledge that many of the *L. hesperus* females showed a marked propensity to deposit eggs, often in clusters, in the beak and around the sepals and calyx of the bean pod. These eggs were often difficult to identify or count, especially immediately after oviposition. Additional effort was focused on these areas of the bean in subsequent repetitions. The improvement noted between the first and subsequent repetitions offers a compelling argument for the conduct of preliminary experiments wherein the adequacy of procedures is assessed. Because subsequent repetitions of the experiment were appropriately described by common regression lines, no improvement in sampler accuracy occurred after the initial repetition. Therefore, a single sampling exercise with shared feedback was sufficient to achieve a stable level of sampler proficiency.

The greatest improvement in sampler performance occurred between the first and subsequent repetitions of the experiment (Fig 1a vs Fig 1c; Fig. 1b vs Fig. 1d), and was attributed to improved searching image. However, improvements associated with increased egg development were not trivial (Fig. 1a vs Fig. 1b; Fig. 1c vs Fig. 1d). The eggs at three days after oviposition were more apparent and easier to count especially when they were deposited in cryptic locations or in clusters. Although the relationship between the zero-d egg counts and total eggs is strong

Table 2. Point estimates and 95% confidence intervals (C.I.) of *L. hesperus* egg numbers from regressions relating counts taken 3 days after oviposition to total eggs for an initial (1) and subsequent (2 – 6) experimental repetitions

Experimental repetition[z]	Total eggs	Model-estimated eggs (± S.E.)	95% C.I.	Estimated % of total eggs
1	5	2.17 (± 0.16)	1.81 — 2.52	43.4
	10	3.52 (± 0.41)	2.61 — 4.43	35.2
	20	6.23 (± 1.05)	3.91 — 8.55	31.2
2 – 6	5	4.70 (± 0.32)	3.41 — 5.99	94.0
	10	9.55 (± 0.34)	8.41 — 10.69	95.5
	20	19.24 (± 0.57)	18.04 — 20.44	96.2

[z] Groupings of experimental repetitions were based on tests of common slopes using ANCOVA.

enough to be of utility, and the confidence intervals are actually smaller than those for the three-d egg counts (Tables 1, 2), the counts of three-d-old eggs demonstrated a higher level of concordance with total eggs compared with the initial counts. In addition, accuracy of the regression predictions for the zero-d egg counts increased with increasing numbers of eggs (Table 1). We attributed this instability in accuracy to behavioral differences among females laying many versus few eggs. Females depositing many eggs tended to use more locations on the bean for oviposition whereas females laying few eggs more frequently deposited those eggs in cryptic locations. In contrast, the accuracy of predictions for counts at three days after oviposition were little changed over the range of observed oviposition (Table 2), primarily because eggs in all locations were more apparent.

One aspect of the study we had not anticipated was the occurrence of false-positive egg identifications. Even though the beans used in our study were carefully selected from lots obtained commercially, microscopic examination often revealed insect injury and eggs of species other than *Lygus*. Perhaps more importantly, Cooper and Spurgeon (2012) observed that many oviposition attempts by *L. hesperus* are unsuccessful. These unsuccessful attempts often leave a visible puncture from the ovipositor, but no egg is present. Also, many successful oviposition events leave the egg deeply embedded in host tissue so that the operculum is barely or not at all visible. It seems probable that many of the false-positive egg identifications in this study were caused by unsuccessful oviposition attempts where presence of an egg was mistakenly recorded. False-positive egg identifications in the initial, zero-d counts were not corrected during the three-d counts. This approach was adopted because we were specifically focused on improved egg detection with increased egg development, and did not wish to confound or offset these estimates by removing false-positives. It seems likely that this decision resulted in some inflation of the slope and its standard error for the regression relating counts at three days after oviposition to total eggs (Fig. 1d). We believe that in the absence of the earlier counts most false-positives would be eliminated because of the increased apparentness of the older eggs.

The range of numbers of total eggs observed in our study easily encompassed estimates of *L. hesperus* daily oviposition from previous reports (Balachandran et al., 2014; Mueller and Stern, 1973; Strong et al., 1970). In fact, the mean numbers of eggs (± S.E.) we counted at zero (4.4 ± 0.54) and three days after oviposition (5.3 ± 0.56) are close to those previous estimates even though our longest oviposition period was eight hours. Therefore, our results should be generally applicable to experiments in which daily oviposition of *L. hesperus* is estimated.

Oviposition by *Lygus* spp. is highly variable, as indicated by our results and reports by others for both *L. lineolaris* (Abel et al., 2010) and *L. hesperus* (Strong et al., 1970). In the presence of this variability, the investigator examining all but the most prominent treatment effects on oviposition must either use relatively large sample sizes or accept low statistical power. Some of this variability can be negated through careful experimental design, but adjustments in experimental design cannot by themselves separate variability in oviposition from variability associated with errors in counting eggs. Although delaying the counting of eggs may not be possible where the host is situated in the field or the oviposition period is too long to permit counts before some eggs hatch, our results clearly demonstrate that counts of partially developed eggs of *L. hesperus* are more accurate than are counts of newly deposited eggs. Utilization of this information will allow for increased precision or reduced cost in studies of *L. hesperus* oviposition.

DISCLAIMER

Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U. S. Department of Agriculture. USDA is an equal opportunity provider and employer.

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