External marking and behavior of early instar *Helicoverpa armigera* (Lepidoptera: Noctuidae) on soybean

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Abstract

Helicoverpa armigera Hübner (Lepidoptera: Noctuidae) is a pest of major agricultural crops, such as soybean and cotton. A better understanding of larval movement is important for its integrated management and resistance management. Studies with neonates through second instar larvae are still limited by the difficulties involving the handling and observation of these instars. Many studies require marking larvae, and most research involving marking is focused on moths. However, our study investigated aspects of larval behavior of the second instar of *H. armigera* on soybean plants. The dyes luminous powder red and Sudan Red 7B were tested as external larval markers. Both dyes successfully marked the larvae for most of 1 stadium (48 h) without deleterious effects, and are useful for short-period behavioral studies. Luminous powder red was selected for the *H. armigera* larval behavior study on soybean because of ease of detection during both day and night. Second instar on-plant movement was consistent, independent of the d period (morning, afternoon, evening). In general, larvae established their feeding site within a few hours of release, and remained feeding on soybean leaves. Second instar behavior suggests that management by nocturnal insecticide application, based on *H. armigera* larval movement, would not have an advantage over daytime application.

Key Words: larval marking tools; dye; old world bollworm; *Glycine max*

Resumo

Helicoverpa armigera Hübner (Lepidoptera: Noctuidae) está entre as principais pragas de importancia para culturas agrícolas, como soja e algodão. Compreender o comportamento larval desta espécie, principalmente durante os estádios iniciais é de suma importância para seu manejo integrado e para o manejo de populações resistentes. No entanto, pesquisas com neonatas ou lagartas de segundo ínstar são limitadas devido às dificuldades envolvendo o manuseio e observação de insetos tão diminutos. Muitos desses estudos requerem a marcação de indivíduos, e até o momento, a maioria das pesquisas com marcação de insetos é focada em adultos. Assim, nosso estudo investigou aspectos do comportamento de lagartas de segundo instar de *H. armigera* em plantas de soja. Estudos prévios também foram realizados com o intuito de se avaliar métodos alternativos e eficazes para marcação de estádios iniciais das larvas desse noctuídeo e suas aplicações em estudos de comportamento. Para tanto, os corantes luminous powder (azul e vermelho) e Sudan (azul e vermelho 7B) foram testados por meio da incorporação em dieta artifical e polvilhamento sobre as lagartas. Baseado em nossos ensaios prévios de laboratório, os corantes incorporados na dieta artifical apresentaram efeitos variáveis sobre os parâmetros biológicos de *H. armigera* e baixa persistência após o segundo ínstar. Os corantes aplicados por polvilhamento marcaram com sucesso as lagartas e luminous powder vermelho foi selecionado para o estudo de comportamento de lagartas de segundo ínstar em plantas de soja. Lagartas de segundo ínstar apresentaram comportamento de movimento nas plantas semelhantes, independentemente do período de avaliação (manhã, tarde e noite). Em geral, a maioria das lagartas estabeleceram seu sítio de alimentação após algumas horas e permaneceram se alimentando sobre as folhas de soja. Os resultados de comportamento de larvas de *H. armigera* em segundo instar, documentado no presente trabalho, indicam que aplicações noturnas de inseticidas não representa vantagem para aumento da eficiência de controle, quando comparado com aplicacões de inseticidas durante o dia.

Palavras Chave: técnicas de marcação larval; corante; lagarta do velho mundo

Helicoverpa armigera Hübner (Lepidoptera: Noctuidae) is one of the major lepidopteran pests of agriculture worldwide, having been detected in South America during the 2012 to 2013 crop season (Czepak et al. 2013; Specht et al. 2013; Murúa et al. 2014). In 2015, specimens were detected in pheromone traps in Florida, USA (Hayden & Brambila 2015), though it is not known to have become established in the country.

The larval stages of this and other lepidopteran species are highly vulnerable to predators, parasitoids, and pathogens (Zalucki et al. 1986, 2002; Johnson et al. 2007; Perkins et al. 2008). Their early movement and dispersal largely determine where feeding sites become established (Zalucki et al. 1986; Pannuti et al. 2016a). Depending on the feeding site, many lepidopterans find shelter that limits the use or efficacy of important control strategies, such as chemical and biological

control. In transgenic crops expressing *Bacillus thuringiensis*-derived proteins (*Bt*), an understanding of larval dispersal is fundamental to resistance management, because larval mobility and selection of feeding sites influence larval exposure to lethal and sublethal concentrations of *Bt* proteins (Burkness et al. 2012; Yang et al. 2014). However, knowledge of the larval lepidopteran feeding and movement behaviors are critical for the design of effective integrated pest management (IPM) and insect resistance management (IRM) strategies (Ross & Ostlie 1990; Spangler & Calvin 2001; Zalucki et al. 2002; Paula-Moraes et al. 2012; Pannuti et al. 2016b).

Although the importance of understanding larval behavior is recognized, studies focusing on early instar behaviors are scarce for noctuids. The nocturnal behavior of most of the species of this family of Lepidoptera, difficulty in handling them (EPPO 1981; Zalucki et al. 2002), difficulty in observing them under field conditions, and the confounding effects of natural insect infestations makes study extremely difficult.

Faced with these limitations, insect marking is a valuable tool for studies examining larval behavior. A wide variety of mark-release-recapture methods have been used for insect studies (e.g., Akey 1991; Southwood & Henderson 2000; Hagler & Jackson 2001), ranging from sophisticated and expensive methods, such as molecular markers or radar, to less expensive approaches, such as abrasion or paints (Warner & Bierzychudek 2009). Dye is an ideal marking material because it is inexpensive, non-toxic, identifiable, requires minimal manipulation, and it is easily applied (Hagler & Jackson 2001; Qureshi et al. 2004; Zhao et al. 2008). Dyes have been successfully applied, internally and externally, to mark the life stages of Lepidoptera in many studies (e.g., Ostlie et al. 1984; Zhao et al. 2008; Vilarinho et al. 2011). External marking by dusting dye is the most popular method used to mark larvae and adults; however, concerns about using dusts have been reported, including the difficulty in getting lasting adherence and disturbances to biological aspects of the insects (Akey et al. 1991). Dyes incorporated into a larval diet have been used to mark insects internally, principally for sterile insect release programs and characterization of insect movement for area-wide integrated pest management and resistance management programs (Shimoji et al. 1999; Stephens et al. 2008; Vilarinho et al. 2011).

Different dyes at different concentrations show variable efficiencies and effects on the development of insect species (Hendricks et al. 1971; Hunt et al. 2000; Qureshi et al. 2004). Dyes do not effectively mark all insects, and not all species can tolerate them; therefore, it is necessary to verify the efficacy of each marker dye for each of the different insect species (Hunt et al. 2000; Qureshi et al. 2004). Most studies using dye have focused on marking adults (Shimoji et al. 1999; Hunt et al. 2001; Stephens et al. 2008), whereas few studies focused on marking early instars.

Considering the lack of information on *H. armigera* behavior in soybean, the objective of this study was to investigate the efficiency and effect of select external marking dyes (luminous powder and Sudan dyes) on early instar *H. armigera*, and behavior of second instar *H. armigera* in soybean.

Material and Methods

The bioassays were carried out in the laboratory at 25 ± 2 °C, 60 ± 10% RH, 14:10 h (L:D) photoperiod, and greenhouse conditions in 2015. The *H. armigera* colony was initiated with insects collected in São Desidério, Bahia, Brazil (12.3600°S, 44.9700°W) during the 2014 to 2015 cropping season from non-*Bt* cotton. The colony was maintained at the same environmental conditions as the laboratory bioassays. A bean-based artificial diet (Parra 2001) was used for rearing *H. armig-*

era. The species was identified by adult genitalia dissection and based on morphological characters (Pogue 2004).

MARKING EFFICIENCY AND EFFECTS OF DYES ON *HELICOVERPA ARMIGERA*

In order to optimize marking techniques for examining *H. armigera* larval behavior on soybean, experiments were conducted comparing the efficiency of external dusting dyes on *H. armigera* larvae. The dyes used to mark the insects were luminous powder red (BioQuip Products, Rancho Dominguez, California, USA) and Sudan Red 7B (Sigma-Aldrich Corporation, St. Louis, Missouri, USA).

First-instar *H. armigera* were monitored until initiation of the second instar (< 6 h), at which point the dust markers were applied. Five insects were marked per treatment with 10 replications (50 individuals in total). The study was conducted using a completely randomized design. Before splitting the larvae into groups, the 50 larvae used for each treatment were placed in a 1 L container and dusted. Approximately 0.5 g of each dye was filtered and dusted onto the group of larvae such that the dye was visible on each larva. After dusting, larvae were separated into groups of 5 and placed into 100 mL plastic cups containing 15 mL of the regular diet, and closed with a plastic lid. Each cup represented 1 replication. The treatments were control (larvae without dusting), Sudan Red 7B, and luminous powder red. The number of marked larvae and their instar were evaluated every 12 h until all the larvae lost the external marking. The external visual inspection for marking was performed under normal light for Sudan Red 7B, and using an ultraviolet (UV) flashlight (Latarka, Ultrafire WF-501B, Ultrafire, Guangdong, China) for luminous powder red. The use of the ultraviolet flashlight did not prove advantageous for detecting Sudan Red 7B, but helped detect luminous powder red.

BEHAVIOR OF SECOND INSTAR *HELICOVERPA ARMIGERA* IN SOYBEAN

This bioassay was conducted under greenhouse conditions at 25 ± 4 °C, 60 ± 10% RH, and natural light. The soybean cultivar 'Conquista' was cultivated in 5 L pots containing autoclaved substrate (soil, sand, and organic matter at a ratio of 1:1:1). The substrate was fertilized as recommended for the crop (Mascarenhas & Tanaka 1997). The plants were individually placed into cages (45 cm D × 65 cm H) and when they reached the R4 to R5 reproductive stage (Fehr & Caviness 1977) they were infested with 15 second instar *H. armigera*. The cages were kept covered with voile fabric during the entire experiment.

Prior to infestation, the larvae were externally marked with luminous powder red dye following the dusting methodology as previously described. There were 5 replications, each composed of 1 plant with 15 larvae. The behavioral evaluations were performed in the morning, afternoon, and evening (6:00–7:00 AM, 2:00–3:00 PM, and 8:00–9:00 PM). The experiment was a randomized complete block design. Each plant was composed of 1 block. The evaluated parameters were the number of observed larvae, larval movement, and feeding site choice. Observing the larvae at different periods (morning, afternoon, evening) during 48 h after infestation also served to document the duration of the marking technique. The number of larvae was calculated by averaging the values of the first 2 d because these d included nocturnal evaluations, and all larvae remained marked for this period. Larval behavior was divided in 2 categories: static (feeding or resting) and dispersing (crawling or ballooning). The feeding site choice also was divided into 2 categories: leaf consumption and pod consumption. The treatment design was a 2 by 3 factorial, which corresponds to 2 larval behaviors (static or dispersing behavior for the larval movement vari-

ables; leaf or pod consumption for the feeding site choice variables), and 3 different periods of the day (morning, afternoon, or evening).

The evaluations were performed until all the larvae lost the external marking. At this time, all larvae were recovered for classification based on the width of head capsules (Butler 1976). Morning and afternoon evaluations were conducted under natural light, and evening evaluations were performed using the ultraviolet flashlight.

STATISTICAL ANALYSES

Data were analyzed by ANOVA and F tests. Normality was verified by a Shapiro-Wilk's test and homogeneity by Levene's test. Data were analyzed using a generalized mixed model (Proc Glimmix) (SAS Institute 2009) to detect differences between means. When appropriate, means were separated using Fisher's least significant differences procedures (α = 0.05).

Results

MARKING EFFICIENCY AND EFFECTS OF DYES ON *HELICOVERPA ARMIGERA*

The persistence of the 2 external dye markers over the 72-h observation period did not differ ($P > 0.05$) among treatments, though the persistence of both dyes diminished with time (Table 1). The percentage of larvae externally marked was 100% for the luminous powder red and Sudan Red 7B dyes up to 24 h after dusting. Although some larvae lost the external marking at 36 and 48 h, the percentage marked remained high for 48 h. Most of the larvae lost the external marking by 60 h after dusting. By 72 h after dusting, no larvae showed any visible external marking (Table 1). There were no significant differences in the onset of the third stadium of *H. armigera* between the treatments (F = 1.18; df = 147,150; P = 0.3094) (Table 1). Luminous powder red was chosen for the behavior study because it allowed more reliable diurnal and nocturnal evaluations (Fig. 1).

BEHAVIOR OF SECOND-INSTAR *HELICOVERPA ARMIGERA* IN SOYBEAN

The plants were infested during the morning on the first sampling date, and it was observed that most larvae lost their external marking by the evening evaluation of the third sampling d. Thus, the data for the morning period on the first sampling d and for the evening period on the third sampling d are not presented in the tables.

The number of larvae observed on the plants was similar for all periods during the second d, and there were no significant differences between the periods (F = 0.25; df = 19,22; P = 0.7817) (Table 2). On average, we observed 13 out of 15 infested larvae on each soybean plant during the first 2 d of evaluation, independent of the observation period.

There was a significant larval behavior difference on all sampling d ($P < 0.05$), where most of larvae were found resting or feeding (static behavior), regardless of the period of the d (Table 3). On the first sampling d, there was a significant interaction between larval behavior and period of the d (F = 58.07; df = 10,18; P = < 0.0001), where the mean number of observed larvae feeding or resting (static behavior) at night (14.25) was higher than the number observed feeding or resting during the afternoon period (9.80). The mean number of larvae crawling or ballooning (dispersing behavior) in the afternoon period (3.80) was higher than during the evening period (0.75) for the same sampling d (Table 3).

There was no significant period effect on mean number of larvae on the second and third sampling d ($P > 0.05$). Also, there was no significant interaction between larval behavior and period of the d for the same sampling d ($P > 0.05$) (Table 3). A significant feeding site effect on all sampling d was observed ($P < 0.05$), and most of the larvae were found feeding on leaves independent of the period of the day (Table 4). There was no significant interaction between the feeding site and the period of the d for the first sampling d (F = 0.12; df = 10,18; P = 0.7341) (Table 4). However, there was a significant period effect on mean number of larvae for the same sampling d (F = 7.99; df = 10,18; P = 0.0180), where the mean number of larvae feeding on leaf structures was significantly higher at night than during the afternoon period (Table 4). There was no significant period effect on mean number of larvae on the second and third sampling d when comparing the feeding sites ($P > 0.05$). There was no significant interaction between the feeding site and period of the d for the same sampling d ($P > 0.05$) (Table 4). The percentage of larvae feeding on tissues varied from 8% to 20% (pods) and 80% to 92% (leaves) during the periods of evaluation (Fig. 2). Most of the recovered larvae on the fourth sampling d were classified as third instar, with a mean head capsule width of 0.76 mm (Table 4).

Discussion

Prior to designing the behavioral study of *H. armigera*, the persistence and effect of dusting markers were evaluated. Higher percentages of larvae marked externally with luminous powder red and Sudan Red 7B dyes were observed up to 48 h. After this period, the larvae started losing the external marking due to molting. These results agree with previous studies (Stern & Mueller 1968; Akey 1991), which found that dusts usually are restricted to 1 life stage. For lepidopteran species, dusting lasts only 1 stadium. Several concerns about using dusting marking have been discussed in the literature, including toxicity and retention (Gangwere et al. 1964). According to Stern and Mueller (1968), the particle size is important in getting dusts to adhere. Disruption of behavioral activities is possible also if the particles are coated too heavily on the integument of small or delicate arthropods (Akey 1991). The dusting method used in the present study showed no negative effect

Table 1. Stadium length (± SE) and percentage of *Helicoverpa armigera* second instar larvae marked (± SE) by dusting with luminous powder red and Sudan Red 7B dyes at different times after dusting under laboratory conditions.

| Treatment | Dust persistence (%) at time intervals after application[a] | | | | | | Mean stadium length (h) |
	12 h	24 h	36 h	48 h	60 h	72 h	
Luminous powder	100.00	100.00	96.00 ± 2.67 a	92.00 ± 4.42 a	10.00 ± 3.33 a	0.00	59.52 ± 0.90 a
Sudan Red	100.00	100.00	90.00 ± 4.47 a	82.00 ± 6.29 a	6.00 ± 3.06 a	0.00	57.36 ± 1.20 a
Control	—	—	—	—	—	—	57.60 ± 1.14 a
P	—	—	0.2643	0.2098	0.3880	—	0.3094

[a]Means within columns followed by the same letter are not significantly different at $P \leq 0.05$.
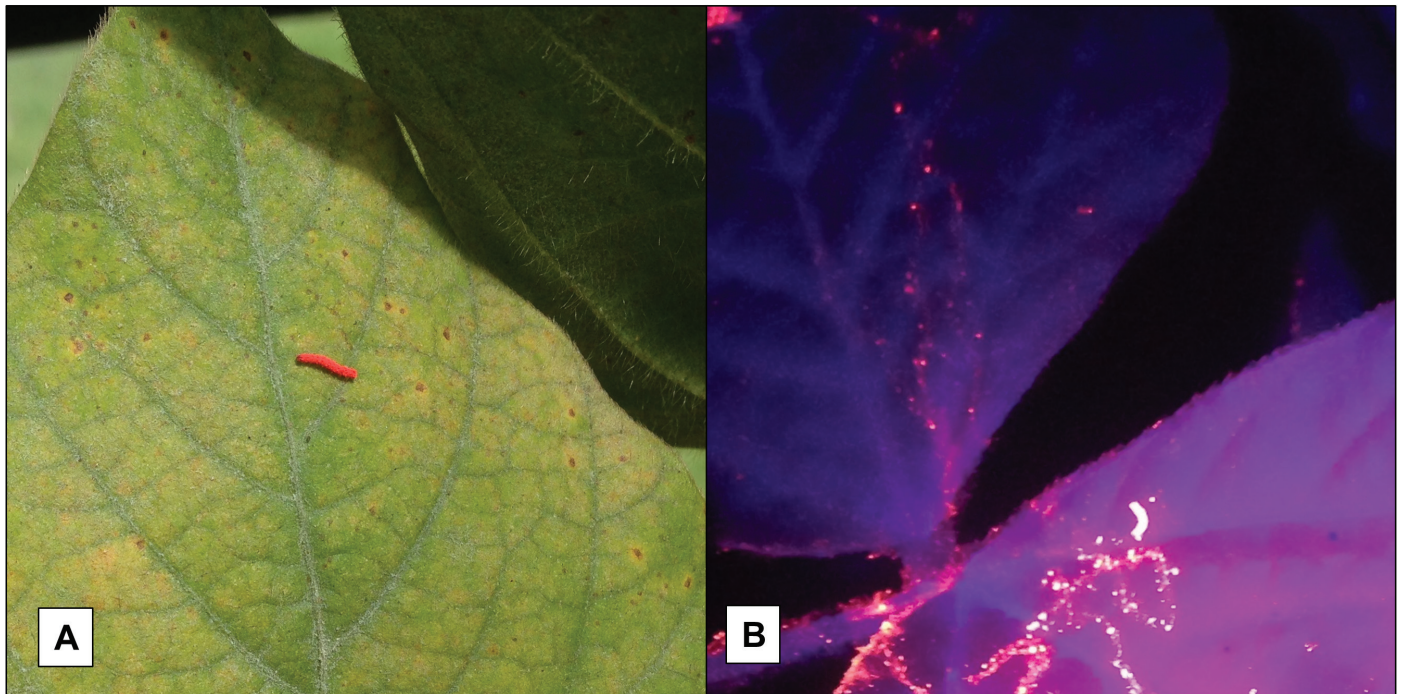
Fig. 1. *Helicoverpa armigera* second instar larvae marked with luminous powder red during diurnal evaluation (A), and its movement and visualization during nocturnal evaluation (B) on soybean plants.

on second-instar *H. armigera*, and the particles successfully adhered to the larvae, which allowed short-term studies.

Twelve h after infestation, most *H. armigera* larvae already were static (feeding or resting) on the different plant tissues. According to the literature, after hatching, neonates shelter for a short period and then enter a "pre-feeding movement phase" (Zalucki et al. 2002). In this study, plants were infested when the larvae turned second instar, so the larvae did not experience the short sheltering period common in neonates, and rapidly entered the movement phase to find a suitable feeding site. A significant number of larvae were dispersing by crawling or ballooning at 12 h after infestation; however, this movement phase seems to be less than 24 h, because almost all the larvae were already established at a feeding site by the evening evaluation (24 h after infestation). A few larvae still were crawling on the plant or ballooning on the other sampling d, but most stayed at the same feeding site, whether nearby or far from the release point, regardless of the period of the d. Considering these results, it can be inferred that after infestation, second instar larvae start dispersing on the plant for a few h until finding a suitable feeding site. After this initial exploration, most of the larvae remain at this established location regardless of the period of the d.

Table 2. Mean (± SE) number of *Helicoverpa armigera* larvae observed on soybean plants in 2 d at 3 different periods of the d.

Period of evaluation	Number of larvae[a]
Morning	13.00 ± 0.82 a
Afternoon	13.00 ± 0.55 a
Evening	12.33 ± 0.91 a
P	0.7817

Periods of evaluation = Morning: 6:00 to 7:00 AM; Afternoon: 2:00 to 3:00 PM; Evening: 8:00 to 9:00 PM. Mean number of larvae from d 1 and 2.
[a]Means followed by the same letter are not significantly different at *P* ≤ 0.05.

Table 3. Mean (± SE) number of static or dispersing *Helicoverpa armigera* larvae on soybean plants at different periods of the d. Botucatu, São Paulo, Brazil.

	Larval behavior[a]	
Evaluation period	Static	Dispersing
D 1		
Morning	—	—
Afternoon	9.80 ± 0.49 bA	3.80 ± 0.49 aB
Evening	14.25 ± 0.48 aA	0.75 ± 0.48 bB
P period	0.1856	
P behavior	< 0.0001	
P interaction	< 0.0001	
D 2		
Morning	10.25 ± 0.85 aA	2.75 ± 0.25 aB
Afternoon	10.00 ± 0.71 aA	2.50 ± 0.50 aB
Evening	9.25 ± 0.48 aA	1.25 ± 0.75 aB
P period	0.1398	
P behavior	< 0.0001	
P interaction	0.8991	
D 3		
Morning	8.25 ± 0.48 aA	0.25 ± 0.25 aB
Afternoon	8.00 ± 0.71 aA	0.25 ± 0.25 aB
Evening	—	—
P period	0.8347	
P behavior	< 0.0001	
P interaction	0.8347	

Periods of evaluation = Morning: 6:00 to 7:00 AM; Afternoon: 2:00 to 3:00 PM; Evening: 8:00 to 9:00 PM. Static behavior represents the larvae that were observed feeding or resting on the plants. Dispersing behavior represents the larvae that were observed ballooning or crawling on the plants.
[a]Means followed by the same letter are not significantly different at *P* ≤ 0.05. Lower case letters refer to comparisons within the column, and capital letters refer to comparisons within the row.

Table 4. Mean (± SE) number of *Helicoverpa armigera* larvae feeding on soybean pods or leaves at different periods of time, average diam of head capsule and estimated instar. Botucatu, São Paulo, Brazil.

Evaluation period	Feeding site[a]	
	Pods	Leaves
D 1		
Morning	—	—
Afternoon	0.80 ± 0.58 aB	9.00 ± 0.84 bA
Evening	2.75 ± 0.85 aB	11.50 ± 0.87 aA
P period	0.0180	
P Feeding site	< 0.0001	
P interaction	0.7341	
D 2		
Morning	1.00 ± 0.71 aB	9.25 ± 0.95 aA
Afternoon	2.00 ± 0.91 aB	8.00 ± 0.71 aA
Evening	1.50 ± 0.65 aB	7.00 ± 1.08 aA
P period	0.8067	
P Feeding site	< 0.0001	
P interaction	0.1752	
D 3		
Morning	1.25 ± 0.25 aB	7.00 ± 0.71 aA
Afternoon	1.25 ± 0.48 aB	6.75 ± 1.11 aA
Evening	—	—
P period	0.8486	
P Feeding site	< 0.0001	
P interaction	0.8486	
D 4	Head capsule width (mm)	Estimated instar[b]
Larvae	0.76	Third

Periods of evaluation = Morning: 6:00 to 7:00 AM; Afternoon: 2:00 to 3:00 PM; Evening: 8:00 to 9:00 PM.

[a]Means followed by the same letter are not significantly different at *P* ≤ 0.05. Lowercase letters refer to comparisons within column, and uppercase letters refer to comparisons within row.

Helicoverpa armigera larvae are thought to be flower, fruit, and seed feeders in preference to leaf feeders (Wilson & Waite 1982; Green et al. 2002; Rajapakse & Walter 2007; Lu et al. 2011). In addition to the higher damage by attacking reproductive plant tissues, such behavior could limit the use of control strategies, because those structures usually provide shelter. However, for this study, most of the larvae were observed feeding on leaves on all sampling d independent of the period (Table 4). It is possible that the second instar larvae were unable to drill the pods and feed on them due to their small size. Larvae of *H. armigera* initially start feeding on tender leaves on whole plants, but eventually they move to the reproductive organs (Liu et al. 2004), which was observed in the present study. The early instar larvae, predominant on leaf tissue, are exposed and might be more susceptible to management methods (such as chemical and biological control). Being exposed could influence the spraying technology methods used to manage the insect populations (e.g., nozzle type). In addition, early larval movement on plants affect their exposure to *Bt* proteins expressed by genetically modified plants, which present different expression of proteins according to the tissue age and structure (vegetative or reproductive) (Yu et al. 2013). The results of this study can be inferred for soybean, which was used as a model. The insect behavior may be different on other important hosts, like cotton.

Insect behavior did not differ from morning to afternoon. This result is not consistent with the recommendation of some researchers and farmers who have been considering spraying pesticides during the night, based on the report of the nocturnal activities of lepidopteran
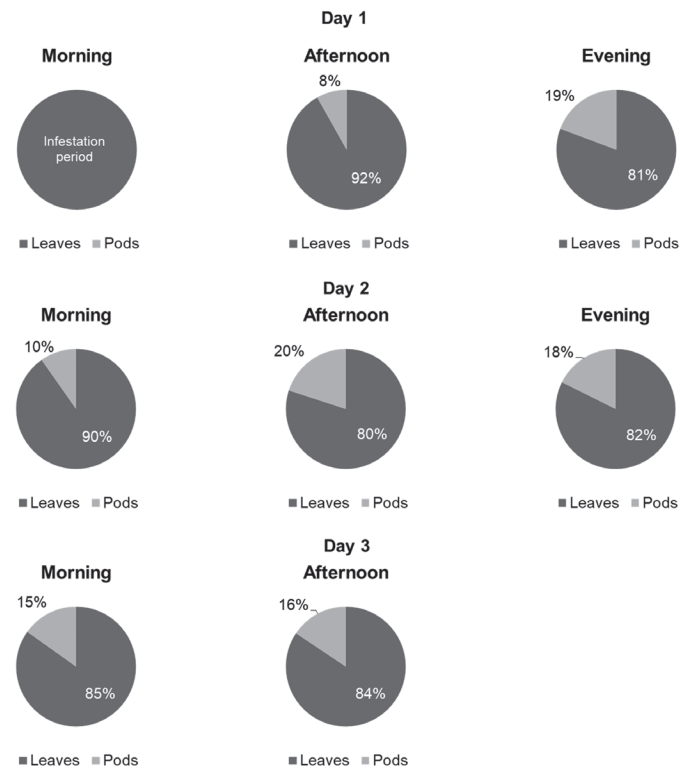
Fig. 2. Percentage of *Helicoverpa armigera* larvae feeding on soybean tissues at different periods of the d. Botucatu, São Paulo, Brazil.

larvae, including *H. armigera* (EPPO 1981). However, ultraviolet radiation associated with sunlight degrades both chemical insecticides and biological insecticides, so there are potential benefits to afternoon or evening applications.

Luminous powder red, the dusting dye used here, was found to be a valuable tool to mark and conduct short-term diurnal and nocturnal studies involving behavior of young lepidopteran larvae. Our study contributes to the understanding of feeding behavior and larval movement of young *H. armigera* in soybean. Characterization of larval behavior will support a more efficient strategy for the integrated pest management and insect resistance management of this pest.

Acknowledgments

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