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Timing and ecdysteroid regulation of the molt in last instar greenhouse whiteflies (*Trialeurodes vaporariorum*)

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Abstract

A system of markers has been devised to track the development of 3rd and 4th instar/pharate adult greenhouse whiteflies. Instars were identified based on measurements of body width and body length. Depending upon the host plant, the product of the two measurements was exceptionally useful in distinguishing between instars. Body depth was used to divide the 3rd instar into eight stages and body depth and color and appearance of the developing adult eye were used to divide the 4th instar/pharate adult into nine stages. Under conditions of L:D 16:8 and a temperature of 26±2°C, the body depth of 3rd instars reared on greenbean increased from 0.025 (stage 1) to 0.2 mm (stage 8) and the instar duration was approximately 3 days. The body depth of 4th instars increased from approximately 0.1±0.02 (Stage 1) to 0.3±0.03 mm (Stage 5) and then remained constant or decreased slightly during adult development. Ecdysteroid titers peaked at approximately 120 fg/μg protein during Stages 3 through 6 of the 4th instar. Based on an external examination of developing 4th instars and the fluctuations in ecdysteroid titer, it appears that adult development is initiated in Stage 4 or 5 4th instars. Results from histological studies support this view. In Stage 4 nymphs, a subtle change was observed in the corneagenous cells of the eye. However, most Stage 4 4th instars possessed wing development characteristic of earlier, immature stages. In all Stage 5 insects, wing development had been initiated and the corneagenous cells had become quite distinct. In Stage 6 whiteflies, the wing buds were deeply folded and by Stage 7, spines were observed on the new cuticle, indicating that the adult cuticle was well-formed by this stage. Our study is the first to investigate the timing and regulation of the molt, to monitor ecdysteroid titers in precisely staged 4th instar whiteflies and to examine the internal anatomical changes associated with metamorphosis in these tiny homopteran insects. Published by Elsevier Science Ltd.

Keywords: Greenhouse whitefly; Staging system; Developmental markers; Ecdysteroids; Molting; Metamorphosis; Morphology

1. Introduction

Whiteflies attack more than 500 species of food, fiber and ornamental plants causing crop losses that total to hundreds of millions of dollars (van Lenteren and Woets, 1988; Costello et al., 1992; Tsujita and Roberts, 1995; Heinz, 1996; Henneberry et al., 1997). Whitefly damage is caused by insects feeding on phloem, by honeydew production which results in the growth of sooty mold, and through the transmission of viruses, many of which either severely damage or kill the host plant (Byrne and Bellows, 1991). Concern about the development of pesticide resistance in whiteflies (Cahill et al. 1994, 1995, 1996a,b) as well as the need to reduce pesticide usage because of environmental considerations has resulted in increased emphasis on the use of cost-effective biological control strategies in IPM programs. However, although there is a growing body of literature concerning the basic biology of whiteflies (Byrne and Bellows, 1991) and the physiology of feeding (Salvucci et al. 1998, 1999), little information is available concerning the endocrine regulation of their development, information that is essential to the enhancement of existing, as well as the development of new biological control technology. Here we present a precise staging system for identifying physiologically synchronous whiteflies, describe the structural changes (internal and external) that accompany the molt from the larva to the adult and track molting hormone fluctuations in last instars of the greenhouse whitefly.

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2. Materials and methods

2.1. Chemicals

Twenty-hydroxyecdysone (20E) was purchased from Sigma (St. Louis, MO, USA). Ecdysone antiserum and the peroxidase-labeled ecdysone conjugate used in the enzymeimmunoassay (EIA) was provided by T. Kingan (University of California at Riverside, USA). The antiserum has a high affinity for ecdysone, 20E, 3-dehydroecdysone, 20,26-dihydroxyecdysone, 26-hydroxyecdysone and makisterone A (Kingan, 1989; Kingan, personal communication). The goat-antirabbit IGG and the enzyme substrate, 3,3′,5,5′-tetramethylbenzidine (TMB), were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA) and American Qualex (San Clemente, CA, USA), respectively. The Coomassie Plus-200 Protein Assay Reagent was purchased from Pierce (Rockford, IL, USA).

2.2. Insect rearing

Insects used in this study were obtained from a laboratory colony maintained at the Insect Biocontrol Laboratory, Beltsville, MD. The *Trialeurodes vaporariorum* colony was housed in a greenhouse containing a variety of plants including greenbean, eggplant, tomato, poinsettia, cotton and salvia. For these studies, greenbean leaves were removed from the plant at the junction of the petiole and stem and placed in 60 ml tubes containing water and 1% Miragro (Miracle-Gro Products, Inc., Port Washington, NY). The leaves were kept in an incubator at 26±2°C, in a light/dark regimen of L:D 16:8 and at a relative humidity of 60–80%. Leaves were infested with greenhouse whiteflies by placing them in a mesh bag containing between 100 and 300 whiteflies for approximately 18 h. After infestation, adult whiteflies were removed and leaves were returned to the incubator.

2.3. Insect staging

2.3.1. Identification of instar

Insects to be staged were examined daily. A small pen mark was placed next to each 1st instar whitefly that was to be tracked, and a map indicating the location of each whitefly on the leaf was prepared. For these experiments, whitefly development on both greenbean and salvia leaves was studied. Using an optical micrometer, length and width were determined daily (between 11:00 A.M. and 2:00 P.M.) and were recorded until adult emergence or until mortality was observed. Since during a given instar, except for the 4th instar, larvae do not grow in length or in width (Hargreaves, 1915), a jump in both length and width indicated that a molt had occurred. This was usually confirmed by the presence of whitefly exuviae.

2.3.2. Development of a staging system for 3rd and 4th instars

Because, in general, insect larvae do not develop synchronously, it was necessary to create a staging system in order to accurately track fluctuations in hormone titer within an instar. Newly molted whitefly larvae are very flat. Since depth increases and reaches a maximum just prior to the larva's molt (Hargreaves, 1915), we developed a staging system based on the depth of the larva. For 3rd instars, late (plump larvae whose lateral bottom edges were slightly raised from the leaf surface) 2nd instar whiteflies were identified/marked either early in the morning (between 8:00 and 9:00 A.M.) or early in the evening (between 5:00 and 6:00 P.M). Whiteflies that were 2nd instars in the morning and had molted to 3rd instars by early evening were considered to be 1/3 day old. Those that were 2nd instars in the evening and had molted to the 3rd instar in the morning were considered to be 2/3 day old. Whiteflies that were plump 2nd instars when examined at 9:00 A.M. and had molted by noon of the same day were considered to be 1/8 day 3rd instars. At least 20 whiteflies were examined at 1/8 (within 3 h of the molt), 1/3, 2/3, 1, 1 1/3, 1 2/3, 2, 2 1/3, 2 2/3, 3 and 3 1/3 days of age. Ages were approximations since the actual time of the molt was not observed. Body depth (the parameter on which our staging system was based) was determined and recorded for each whitefly. For each time period (1/3 day), the percent of each stage (criteria can be found in Section 3, Table 2) present was determined.

For 4th instars, late (plump) 3rd instars were identified and a similar procedure was followed. In addition, the appearance of the eye [its change from a small, intense red dot, to a diffuse dot, to a light red adult eye, to a medium bipartite red and finally dark red or red–black eye (changes associated with pharate adult development)] also formed the basis of our staging system. Since the duration of the 4th instar was much longer (approximately 9 days) than that of the 3rd instar (approximately 3 days), results for the 4th instar are presented as percent of each stage on a given day rather than on a given third of a day.

2.4. Sampling and extraction of T. vaporariorum for ecdysteroid determination

Staged 4th instars/pharate adults were removed from the leaf and placed in 1.5 ml microcentrifuge tubes (Kontes #749510-1500, Vineland, NJ) containing 75 μl of ice cold 75% aqueous methanol. Whiteflies were homogenized using matching pestles, pestles were washed with an additional 75 μl of 75% methanol and tubes were centrifuged for 5 min at 4°C and 14,000×*g*. Supernatants were transferred to 6×50 mm borosilicate glass tubes and placed in crushed ice. Precipitates were again extracted with 75 μl of 75% methanol, vortexed

and centrifuged. Supernatants were added to their respective tubes and tubes were stored in the freezer at −10°C. An EIA (Kingan, 1989) was used to determine the ecdysteroid content of each sample. Briefly, wells of a 96-well costar #3569 microtiter plate (Corning Inc., Corning, NY) were coated with goat-antirabbit IGG and incubated overnight at room temperature. The IGG was discarded, wells were incubated for 1 h with blocking buffer and then rinsed with phosphate-buffered saline (PBS-Tween). Fifty microliters of appropriately diluted 20E standard in duplicate (range of the assay is 500–40,000 fg) or of sample [dried in a Savant Speedvac Concentrator (Forma Scientific, Marietta, OH, USA) and reconstituted in EIA buffer] was added to a given well. This was followed by the addition of 50 μl of ecdysteroid antibody, and 50 μl of peroxidase-labeled ecdysteroid conjugate. Plates were incubated overnight in the refrigerator at 4°C. Contents were discarded and wells were again washed with PBS-Tween. After color development with TMB and H_2O_2, absorbance was measured using an ELISA plate reader (λ=450) equipped with an IBM-PC compatible microcomputer. Using the data analysis program, SOFTMAX, fg of ecdysteroid present in each sample was calculated from the standard curve (semi-log plot with fg ecdysteroid plotted on the log scale).

2.5. Sampling and extraction of T. vaporariorum filter chamber/gut complexes and greenbean phloem for ecdysteroid determination

Since plant tissues contain ecdysteroid (reviewed in Horn and Bergamasco, 1985), and since whiteflies are phloem feeders, phloem from greenbean plants was titered for EIA-detectable ecdysteroid. Using a 1–5 μl graduated micropipette, 1–4 μl of phloem was collected from the distal end of a petiole that had been severed from the plant using a sharp scalpel (Girousse et al., 1991). Extraction and determination of ecdysteroid concentration was performed as described in the previous section.

In order to determine if the contents of the gut contributed to whole body ecdysteroid titers, filter chamber/midgut complexes, sometimes with hindgut attached (see Weber, 1935 for anatomy) were carefully dissected from Stages 2 and 5/6 greenhouse whiteflies, rinsed in saline and placed in 10/microcentrifuge tube in 75 μl of ice cold 75% aqueous methanol. Homogenization, extraction and determination of ecdysteroid titers were performed as described in the previous section.

2.6. Sampling and extraction of T. vaporariorum for protein determination

Appropriate numbers of staged whiteflies were removed from greenbean leaves and placed in 1.5 ml microcentrifuge tubes containing 75 μl of sodium acetate buffer (0.05 M, pH 5.3). Whiteflies were homogenized and the pestle was washed with an additional 75 μl of buffer. Tubes were sonicated in a water bath for 30–60 s, and then centrifuged for 10 min at 4°C and 14,000×*g*. Aliquots of supernatants were transferred to wells of a 96-well microtiter plate, and sufficient buffer was added to bring the volume to 150 μl. Soluble protein was determined using the Pierce Coomassie Plus Protein Assay (Microwell Plate Version, micro protocol, working range 1–25 μg/ml). Standards were prepared from BSA. Absorbance (λ=595) was determined using an ELISA plate reader. Micrograms of protein present in each sample was calculated from a standard curve (log–log plot).

2.7. Statistical analysis

One way analysis of variance (ANOVA) was used to analyze the ecdysteroid/protein titer data. When *F*-tests were significant, the Fisher's Least Significant Difference (LSD) Comparison of Means Test was used to analyze for significant differences among the various groups, P=0.05.

2.8. Histological methods

Whitefly nymphs were fixed for 2–3 h in Carnoy's Formula 2; 60% absolute ethanol; 30% chloroform; 10% glacial acetic acid (Davenport, 1960). The fixed nymphs were rinsed with absolute ethanol, stained with 1% eosin b in absolute ethanol for 30 min, then washed with absolute ethanol to remove free eosin; this step stains the nymphs pink, allowing them to be more easily manipulated during embedding. The dehydrated nymphs were transferred through four changes of xylene and then placed in paraffin (Paraplast Xtra) at 60°C where they remained overnight. The whiteflies were then transferred to fresh paraffin in embedding molds, and chilled rapidly in ice water.

The embedded nymphs were sectioned at 5 μm on a rotary microtome. Sections were relaxed on water at 40°C, mounted on egg albumin-coated slides, dried, and placed horizontally in a drying oven at 40°C overnight.

Mounted sections were deparaffinized in three changes of xylene, transferred through two changes of absolute ethanol and rehydrated through a series of aqueous ethanol solutions (95, 90, 70 and 50%). Sections were stained with Weigert's iron hematoxylin followed by Casson's trichrome as described by Kiernan (1990)

3. Results

3.1. Using length and width measurements to distinguish among instars

The mean length and width as well as the ranges for length and width for each of the four instars of the green-

house whitefly (reared on greenbean) are shown in Fig. 1(A). For instars 1 and 2, the ranges for length overlap while the ranges for width do not. For 2nd and 3rd as well as 3rd and 4th instars, neither the ranges for length nor the ranges for width overlap. Importantly, the ranges of the products of the length and width for 1st and 2nd instars do not overlap (Table 1), and for 2nd and 3rd as well as 3rd and 4th instars, the use of the product provides better separation than that of either length or width measurements alone (Fig. 1(A) and Table 1). Mean length and width as well as the ranges for the length and width of greenhouse whitefly reared on salvia are provided in Fig. 1(B). All instars of greenhouse whitefly reared on salvia were smaller than their counterparts reared on greenbean. In addition, for both length and width measurements, there was considerable overlap between consecutive instars. However, when products (length×width) were compared, the overlap was reduced considerably (Fig. 2). Thus, overlap between 1st and 2nd instars reared on salvia was reduced from 11 to 2%, overlap between 2nd and 3rd instars, from 10 to 0% and overlap between 3rd and 4th instars, from 11 to 5%.

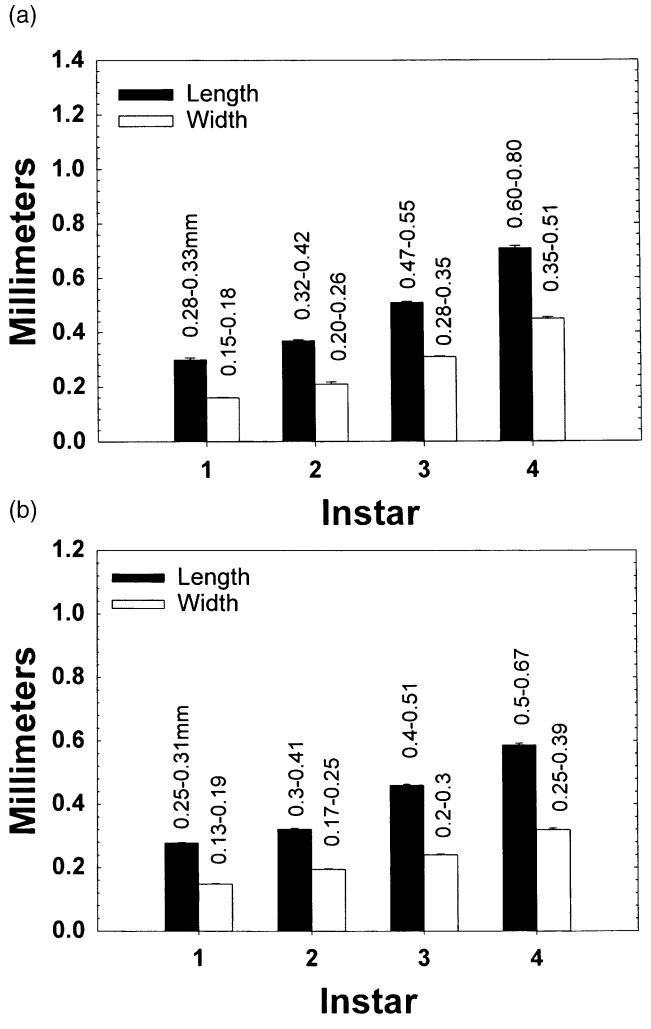

Fig. 1. Mean length and width of the four instars of the greenhouse whitefly. (a) Greenhouse whiteflies reared on greenbean; (b) greenhouse whiteflies reared on salvia. Individual whiteflies were marked and examined daily. An optical micrometer was used to determine the length and width measurements of each whitefly. Data were recorded until the whitefly emerged or mortality was observed. A jump in both length and width indicated that a molt had occurred. Each value represents the mean±S.E. for at least 50 separate determinations. The ranges of length and width measurements are provided above each bar.

Table 1
Ranges for the (length×width) parameter for the four instars of the greenhouse whitefly (whiteflies were reared on greenbean plants). For each instar, a minimum of 50 greenhouse whiteflies were examined

Instar	Length×width (mm^2)	
	Minimum	Maximum
1	0.042	0.0585
2	0.07	0.0955
3	0.14	0.187
4	0.21	0.408

3.2. Staging systems for 3rd and 4th instar greenhouse whiteflies

As the 3rd instar developed, its depth increased and was observed to range between 0.025 and 0.2 mm. Stage assignments were based on depth as shown in Table 2. With the exception of Stages 1 and 8, the median value for each stage was a multiple of 0.025 mm. Typically, under the rearing conditions used, the duration of the 3rd instar was approximately 3 days with 91% of the 3rd instars having molted to the 4th instar by day 3 and 100% having molted by Day 3 1/3 (Table 3). Table 3

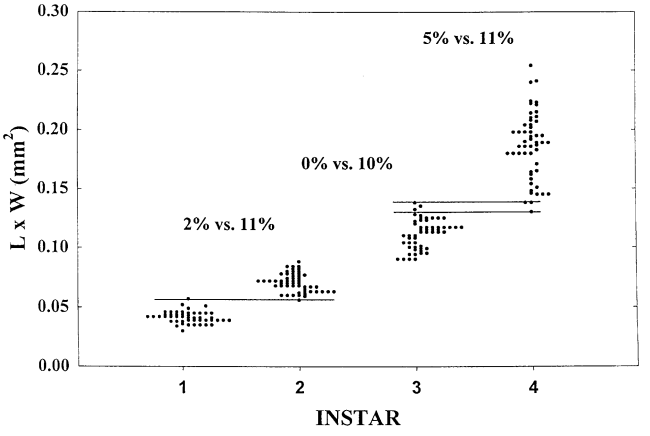

Fig. 2. Distinguishing among the four instars of the greenhouse whitefly based on the product of whitefly length×width. Whiteflies were reared on salvia. Methods as in the legend to Fig. 1. Each point represents the product of length×width for a single whitefly. Horizontal lines identify the overlapping values. For adjacent instars, the first percentage value is the percent of overlap based on product values and the second percentage value is the percent of overlap based on length measurements.

Table 2
Depth measurements for stages of 3rd instar greenhouse whiteflies

Stage	Depth (mm)
1	0.025–0.035[a]
2	0.050±0.01
3	0.075±0.01
4	0.100±0.01
5	0.125±0.01
6	0.150±0.01
7	0.175±0.01
8	0.19–0.2[a]

[a] Ranges of actual measurements.

Table 3
Stage as related to day of the 3rd Instar of the greenhouse whitefly (reared on greenbean at 26±2°C, L:D 16:8. Day 1/8=whiteflies examined within 3 h of the molt. At least 20 greenhouse whiteflies were examined at each fraction of a day, i.e. 1/8, 1/3, 2/3, etc.)

	Stage (%)								
Day	1	2	3	4	5	6	7	8	M[a]
1/8	27	55	18						
1/3	6	55	33	6					
2/3		13	33	54					
1				64	18	18			
1 1/3				33	60	7			
1 2/3				33	50	11	6		
2				17	8	25	33	17	
2 1/3				15	0	46	16	23	
2 2/3					14	43	9	0	33
3						8			92
3 1/3									100

[a] M=Molt to the 4th instar.

also shows the progress of development of 3rd instars as a function of day post-molt. By day 1, the majority (64.7%) of 3rd instars had progressed to stage 4, and by day 2, the majority (25 and 33.3%, respectively) had reached stages 6 and 7.

As the 4th instar developed its depth also increased. Depth ranged between 0.08 and 0.34 mm (Table 4). For Stages 1–5, the eye appeared as a red pinpoint and stage numbers were based on depth. For the first four stages, stage numbers were assigned at 0.05±0.02 mm intervals with Stage 1 equal to 0.1±0.02 mm (Table 4). For stage 5, depth ranged between 0.27 and 0.33 mm. For stages 6 through 9, the depth remained relatively constant (between 0.23 and 0.34 mm with most whiteflies ranging between 0.25 and 0.3 mm), and color and appearance of the eye served as the basis for staging (Table 4, Fig. 3). When the eye pigment becomes diffuse, whiteflies have entered Stage 6. As the whitefly progresses through Stages 7–9, the eye becomes larger and darker. The approximate duration for each stage is also provided in Table 4. Durations were determined by subtracting the

Table 4
Stages of 4th instar/pharate adult greenhouse whiteflies (T=26±2°C, L:D 16:8, Plant=greenbean. Body thickness defines Stages 1 through 5; eye appearance defines Stages 6 through 9)

Stage	Body thickness (mm)	Eye development	Duration (days)
1	0.08–0.12	Red pinpoint	2.0
2	0.13–0.17	Red pinpoint	1.0
3	0.18–0.22	Red pinpoint	1.0
4	0.23–0.26	Red pinpoint	1.0
5	0.27–0.33	Red pinpoint	Combined
6	0.23–0.34	Slightly diffuse	1.5
7	0.23–0.34	Light red, maximally diffuse	Combined
8	0.23–0.34	Medium red, bipartite	1.0
9	0.23–0.34	Blackish red, bipartite	1.5

mean day of a given stage from the mean day of the following stage. The progress of development of 4th instar as a function of day post-molt is shown in Table 5. The duration of the entire 4th instar was approximately 9 days.

3.3. Soluble protein content of 3rd and 4th instar greenhouse whiteflies

Typically, hormone/metabolite concentration is expressed in units/μl hemolymph or units/unit body weight. Since the collection of hemolymph is impractical and the determination of larval weight is also difficult, ecdysteroid content was normalized against total protein content, as well as expressed on a per whitefly basis. The mean amount of soluble protein for each stage of 4th instar/pharate adult greenhouse whiteflies was determined (Table 6). Soluble protein content of staged 3rd instars was also measured. In 3rd instars, protein content increased between Stages 1 and 5, remained the same in Stages 5 and 6, and then increased again reaching its highest level in Stage 8 whiteflies. For 4th instars, protein content also increased between Stages 1 and 5, plateaued between Stages 5 and 8, and then increased in Stage 9 whiteflies.

3.4. Whole body ecdysteroid fluctuations during the development of 4th instar/pharate adult greenhouse whiteflies

Ecdysteroid titer (expressed as fg/whitefly including the filter chamber/gut complex) increased gradually during Stages 1 through 5, peaked significantly at Stages 4

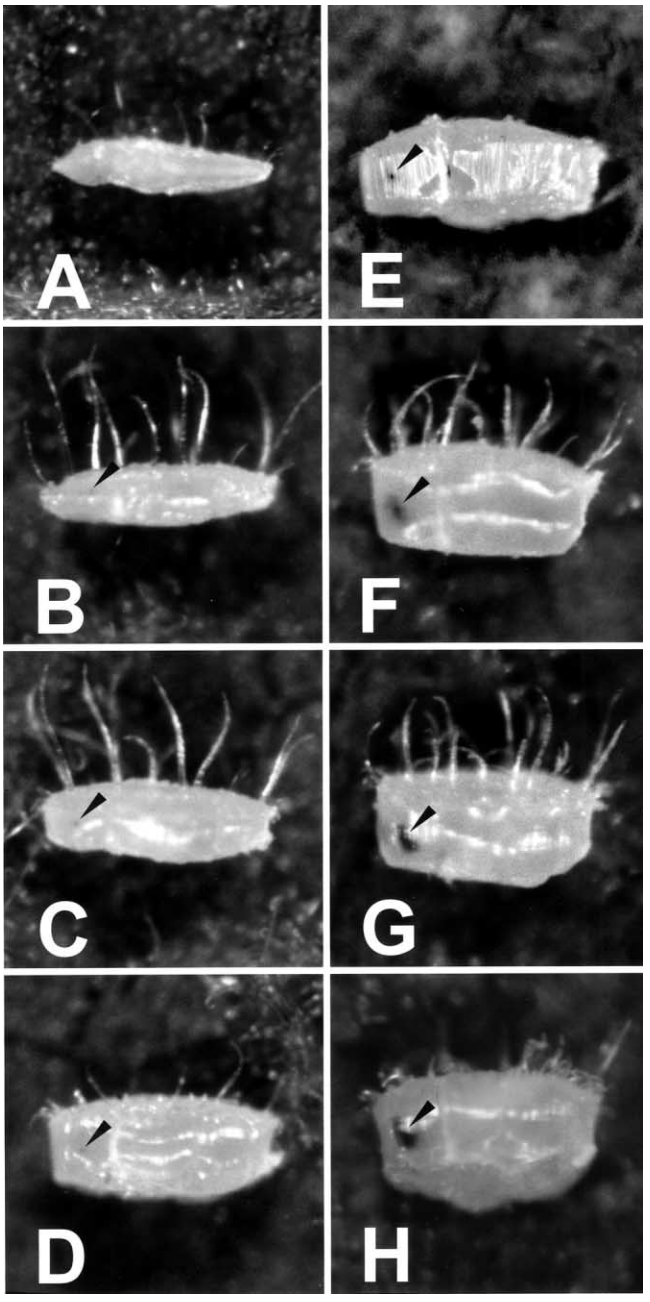

Fig. 3. Stages of the 4th instar/pharate adult greenhouse whitefly. Body depth [for Stage 1 (not shown) and Stages 2 through 5, (A)–(D), respectively] and the color and appearance of the developing adult eye (Stages 6 through 9, (E)–(H), respectively) were used to divide the 4th instar/pharate adult into nine stages (see Table 1 for details). The length of the pointer (indicates location of the eye) = 0.12 mm.

and 5 and then decreased gradually during the rest of the instar (Fig. 4(A))

3.5. Ecdysteroid concentration of greenhouse whitefly filter chamber/gut complexes and of greenbean phloem

The ecdysteroid content of an individual gut complex was approximately 63 fg/gut and did not differ significantly for Stages 2 and 5/6 4th instar/pharate adults (Table 7). Thus, the contribution of the gut to whole body whitefly ecdysteroid titers was more pronounced in early and late 4th instar stages than in the mid-stages when ecdysteroid titers peaked (Table 7 and Fig. 4). In Stages 1 and 2, ecdysteroid in the gut was primarily responsible for EIA-detectable ecdysteroid, and in Stages 3–7 for 20–30% of the activity. The ecdysteroid content of greenbean phloem collected from the leaf petiole was quite low, only 17 fg/4 nl of phloem (Table 7). Based on body weight of whitefly 4th instar/pharate adults (10–45 μg, unpublished results), it was estimated that less than 4 nl of phloem is present in the whitefly gut/filter chamber complex. Therefore, of the ecdysteroid present in this complex, the phloem contributes less than 17 fg.

Table 5
Stage as related to day of the 4th Instar of the greenhouse whitefly (reared on greenbean at 26±2°C, L:D 16:8. Day 0=the day of the molt to the 4th instar; whiteflies were examined within 8 h of the molt)

	Stage (%)									
Day	1	2	3	4	5	6	7	8	9	E[a]
0	100									
1	75	25								
2	19	54	27							
3	10	33	47	10						
4		14	46	20	17	3				
5			9	11	9	40	17	14		
6							5	80	15	
7							13	62	25	
8									70	30
9									20	80
10										100

[a] E=Emergence.

Table 6
Soluble protein content of 3rd and 4th instar greenhouse whiteflies

Stage	μg Protein/greenhouse whitefly[a]	
	3rd Instar	4th Instar
1	0.055±0.004 (e)	0.617±0.13 (E)
2	0.099±0.03 (e)	0.842±0.13 (DE)
3	0.145±0.03 (de)	1.396±0.12 (D)
4	0.240±0.03 (cd)	1.998±0.19 (C)
5	0.307±0.03 (bc)	2.386±0.07 (BC)
6	0.310±0.04 (bc)	2.251±0.22 (BC)
7	0.394±0.04 (ab)	2.677±0.31 (B)
8	0.471±0.06 (a)	2.494±0.17 (BC)
9	–	3.837±0.41 (A)

[a] Each value represents the mean±S.E. for at least six separate determinations except for Stage 1 of the 3rd instar for which the mean was calculated from two determinations. Each sample contained between 3 and 20 greenhouse whiteflies. Means followed by the same letter(s) are not significantly different.

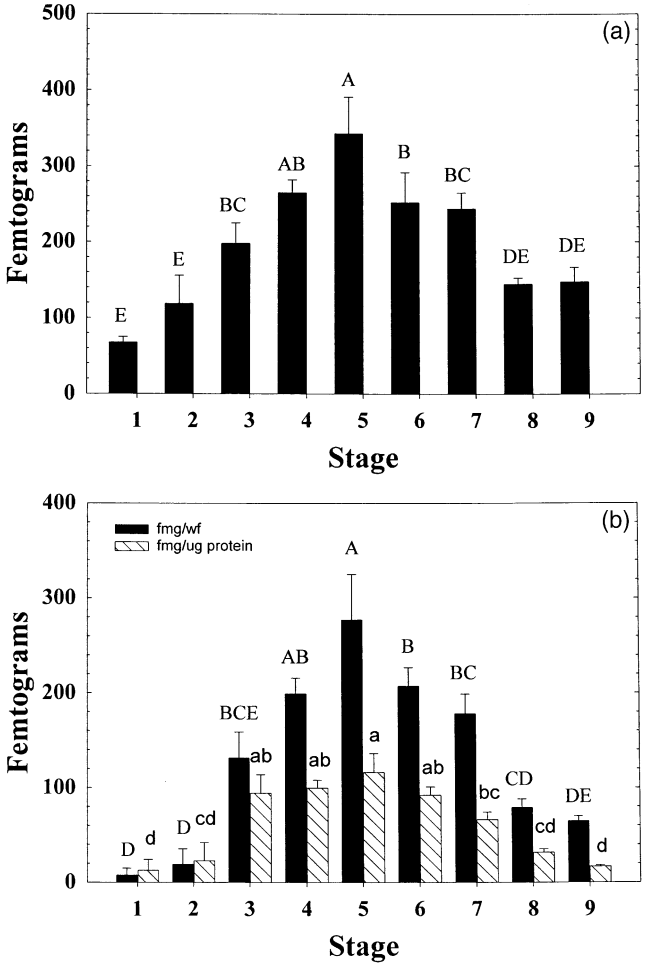

Fig. 4. Whole body ecdysteroid fluctuations during the 4th instar. (a) Including the contribution of the filter chamber/gut complex; (b) with the contribution of the filter chamber/gut complex removed. Whiteflies were extracted in aqueous methanol and titers were determined using an ecdysteroid enzymeimmunoassay as described in Fig. 2. Titers are expressed as fg 20E equivalents per whitefly ((a) and (b)) and also in (b) as fg/μg protein. Each bar represents the mean±S.E. of at least five separate determinations. Means having the same letter designation were not significantly different.

3.6. Whole body (minus filter chamber/gut complex) ecdysteroid fluctuations during the development of 4th instar/pharate adult greenhouse whiteflies

Since the ecdysteroid content of Stages 2 and 5/6 4th instar/pharate adult greenhouse whitefly gut/filter chamber complexes was 70 and 55 fg/gut, respectively, values that were not significantly different (Table 7), a mean value of 62.5 fg was subtracted from whole body ecdysteroid determinations in order to approximate ecdysteroid levels within the whitefly body, i.e. remove the influence of the filter chamber/gut content. Ecdysteroid titer expressed as fg/whitefly or fg/μg protein again increased gradually during Stages 1 through 5, peaked at Stages 4 and 5 and Stages 3 through 7, respectively, and then decreased gradually during the rest of the instar (Fig. 4). Since the filter chamber/gut complex composes a relatively small percentage of the whitefly body, values for soluble protein content provided in Table 6 were used in the latter calculation.

Table 7
Ecdysteroid content of greenhouse whitefly midgut/filter chamber complexes and of greenbean phloem

Stage of 4th instar	fg/gut Complex[a]
2	70±11
5–6	55±7
Source of phloem	fg/4 nl Phloem[b]
Base of petiole	17±2

[a] Each value represents the mean±S.E. for at least three separate determinations. Each sample contained 10 gut/filter chamber complexes.

[b] Since 4th instars weigh between 10 and 40 μg/whitefly (unpublished results), 1–4 nl is approximately 10% of the body volume of 4th instar whiteflies and is probably an overestimation of the fluid content of one whitefly gut/filter chamber complex. Each sample contained approximately 0.125 μl of phloem. The value represents the mean±S.E. for six separate determinations.

Fluctuations in ecdysteroid content of 3rd instars were also monitored (results not shown). Since we were unable to determine the ecdysteroid content of filter chamber/gut complexes of these nymphs, whitefly ecdysteroid levels without the contribution of the gut complex could not be ascertained. However, ecdysteroid content, although not significantly different in Stages 1–4, exhibited a significent drop at Stage 5, from approximately 550 fg/greenhouse whitefly or 3000 fg/μg protein to 75 fg/whitefly or 450 fg/μg protein, respectively.

3.7. Histological examination of staged 4th instar greenhouse whiteflies

The most reliable indicators of the onset of adult development, histologically, were morphological changes of the eye and wing (Fig. 5). Our nomenclature for the eye follows that of Snodgrass (1935). The eye of Stage-3 nymphs was relatively undeveloped; the corneagenous cells that ultimately give rise to the cuticular cornea were indistinct, and the retina was undeveloped (Fig. 5(A)). At Stage 4, a subtle change was observed; the corneagenous cells were more easily discerned, having a somewhat square appearance (Fig. 5(B)). By Stage 5, the corneagenous cells were quite distinct, assuming a more rounded appearance. Also, the retinal cells began to lengthen axially (Fig. 5(C)). By Stage 6, the appearance of the eye had changed radically; the corneagenous cells were distinctly rounded, and the retinal cells had lengthened substantially (Fig. 5(D)). It should be noted that it is in Stage 6 4th instars that diffusion of eye pigments is visible externally. In Stage 7, the cornea was

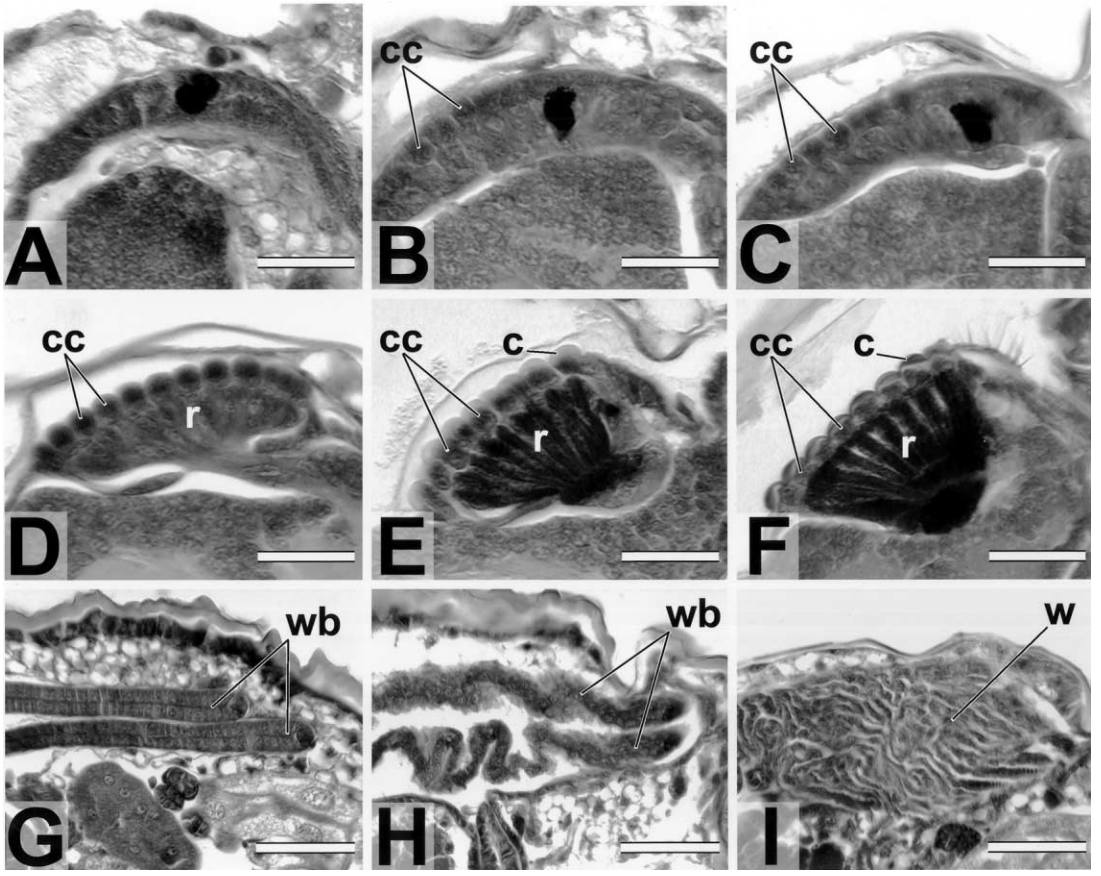

Fig. 5. Histological sections of selected stages of 4th instar/pharate adult greenhouse whiteflies. (A) through (F) depict eye development for Stages 3 through 8, respectively. The bar in the lower right of each photo is equal to 25 μm. (G) through (H) track wing development for Stages 4 through 6, respectively. The bar in the lower right of each photo is equal to 50 μm. cc=corneagenous cells, c=cornea, r=retina, wb=wing bud, w=wing. See Section 2 for histological methods.

first observed over the corneagenous cells, a clear indication of adult cuticle development (Fig. 5(E)). Also commonly observed were proprioceptive hairs posterior to the eye. Retinal pigments were observed at this stage in many, but not all, preparations. By Stage 8, it appeared that the adult eye was fully developed (Fig. 5(F)).

In Stage 3, the wing buds were nearly linear in horizontal section and were composed of a distinct bilayer of columnar epithelial cells. Most Stage 4 nymphs (Fig. 5(G)) had wing buds that were similar to Stage 3; however, in some Stage 4 and all Stage 5 nymphs (Fig. 5(H)), dramatic changes were observed. The wing buds were no longer linear structures but were convoluted. The cells rounded or flattened, and the highly ordered organization of cells into a bilayer was no longer distinct. The growth and expansion of the wings observed by Stage 6 (Fig.5(I)) could be characterized as explosive. In Stage 6 nymphs, the wings were nearly of adult proportions. Indeed, Stage 7 and 8 wings appeared to be little changed in size from Stage 6 wings, the only new developments being the appearance of small cuticular spines on the wing surfaces (results not shown). Also apparent, beginning at Stage 6 was the rapid development of flight muscle in the thorax.

4. Discussion

New or improved systems that are more precise than previously used techniques have been developed for identifying the instar of a greenhouse whitefly and for staging 3rd instar and 4th instar/pharate adult whiteflies. Whitefly appearance including size is known to vary depending upon the identity of the host plant (Mound, 1963; Bethke et al., 1991; Rosell et al., 1995; Neal and Bentz, 1999). Thus, under similar environmental conditions, we observed that all instars of the greenhouse whitefly reared on greenbean were larger than those reared on salvia. Importantly, for both length and width measurements, the ranges of the instars were rather broad. Typically, only mean lengths and widths for a given instar reared on a particular host plant have been reported (Hill, 1969; Nechols and Tauber, 1977; Vet et al., 1980; Bethke et al., 1991), and since the ranges for length and width are relatively broad, use of these means

(typically only length measurements are used) is usually insufficient for identifying the instar of a given whitefly. Our results indicate that for greenhouse whiteflies reared on greenbean, width measurements may be a useful criterion for identifying an instar, but that the product of length×width provides an even better method for identification. For whiteflies reared on salvia, the product of length×width can either reduce or eliminate overlap between adjacent instars. It appears, that for all plants, but especially for plants such as salvia in which greenhouse whiteflies are relatively small and there is overlap in the length and width measurements of consecutive instars, the product of length×width is the best method for identifying the instar of this whitefly species. For *Bemisia argentifolii*, the product of length×width is also exceptionally useful for distinguishing between consecutive instars, especially between the 1st and 2nd instar (unpublished results).

We are not aware of any information in the literature concerning a staging system for any of the first three whitefly instars. Since during each of the first three instars, whiteflies do not increase in length or width, but rather in depth, in these studies the 3rd instar was divided into 8 stages based on depth, the range of which was 0.025–0.2 mm (Table 2). On any given day (day 0=1/8 and 1/3 day, day 1=2/3, 1 and 1 1/3 days, day 2=1 2/3, 2, 2 1/3 days or day 3=2 2/3, 3 and 3 1/3 days), 5 different stages or 4 stages plus the new 4th instar were present. On day 3.0, 8% of the nymphs were at Stage 6 and 92% had molted to the 4th instar. There are at least two possibilities to explain the lack of Stage 7 and 8 nymphs at this time. It is possible that some larvae never enter Stage 7 or 8 and molt after reaching Stage 6. It is also possible that a few insects were developing slowly because they were destined to die. In this experiment, the eventual fate of any given individual whitefly nymph could not be ascertained.

Previously, the 4th instar had been divided into only 3 stages, 'early' (flattened, translucent to opaque-whitish), 'transitional' (expanded, wax-ensheathed, opaque-white with dorsal and lateral, waxy, spine-like processes), and pharate adult (transitional, but with red eyes and yellow body pigment of developing adult visible) (Nechols and Tauber, 1977). In our system, the 4th instar has been divided into 9 stages with the last two of the above 3 stages having been further subdivided. Waxy spines were first observed in Stage 2 and adult eye development as evidenced by the eye's red pigment becoming slightly diffuse occurred at Stage 6. Since for days 2 through 7, there were anywhere between 3 and 6 stages present, staging by age would not be prudent. Dividing the instar into only 3 stages would prevent the detection of important physiological/biochemical changes that occur during the development of the 4th instar and the pharate adult.

Total ecdysteroid titer had to be measured using an EIA whose sensitivity was 100 times greater than that of our ecdysteroid RIA (Gelman et al., 1997). On a per whitefly basis, ecdysteroid peaked at approximately 350 fg in Stages 4 and 5 of the 4th instar. The amount of ecdysteroid in the gut complex of 4th instars did not vary much between Stages 2 and 5/6 and it was assumed that it would be similar in Stages 1 and 7 through 9. Therefore, the gut contributes more to whole body ecdysteroid titers early and late in the instar making the peak even more pronounced in Stage 4 and 5 4th instars. Since the phloem contains such a tiny amount of EIA-detectable ecdysteroid, it should not be the phloem itself, but rather a sequestration phenomenon of the gut or the gut's ability to metabolize ecdysteroids to forms that have greater affinity for the EIA antibody that is responsible for the levels of gut ecdysteroid observed. Expressed as fg/μg protein, the peak broadens to include Stages 3–6, although titers remain highest at Stage 5. In the only other homopteran in which ecdysteroid titers have been tracked, the aphid, *Acyrthosiphon pisum*, ecdysteroid titers for 3rd and 4th instars were determined for hemolymph extracts (Pennacchio et al., 1995) and therefore it is difficult to compare the absolute titers in the two species. Titers between 4 and 8 pg/mg were observed for *A. pisum*, and titers were not significantly different in 3rd and early 4th instars of this aphid (Pennacchio et al., 1995). The maximum titer observed in 4th instar greenhouse whitefly was 116 fg/μg protein.

The fluctuation in ecdysteroid titer was as expected since in other orders of insects for which ecdysteroid levels have been determined, titers are usually low in the early part of an instar and then a premolt ecdysteroid peak is observed just prior to the molt (reviewed by Riddiford and Truman, 1978; Smith, 1985). It is important to note that ecdysteroid EIAs and RIAs measure total ecdysteroid titer and cannot provide information as to the titer of individual ecdysteroids. Thus, it is possible that fluctuations of physiologically active ecdysteroid, typically 20E in almost all orders examined (reviewed by Smith, 1985) [although makisterone A has been reported to be the molting hormone in some hemipterans and in honey bee pupae (Feldlaufer et al., 1985; Kelly et al., 1985)], and of total ecdysteroid are different. For homopterans, the identity of the molting hormone is not known.

Based upon external observation, the first sign of adult development, i.e. the eye pigment becoming diffuse, was observed in Stage-6 individuals. It is reasonable to assume that changes associated with adult development that are invisible to the naked eye occurred prior to this stage. Soluble protein content/whitefly increased through Stage 5 and then remained relatively constant through Stage 8 (Table 6). Generally insects cease to feed and hence to grow just prior to or upon the initiation of a molt, i.e. apolysis. However, whiteflies have been reported to feed during the pharate adult stage (Lie et

al., 1996; Costa et al., 1999). In 3rd instars, soluble protein content also increased through Stage 5 and then remained relatively constant through Stage 7. Therefore, apolysis probably occurred before Stage 5. The precipitous drop in whole body ecdysteroid content between Stages 4 and 5 supports this view, for it is unlikely that the ecdysteroid contribution of the filter chamber/gut complex could differ very much between adjacent stages. Interestingly, in both 3rd (Stage 8) and 4th (Stage 9) instars, soluble protein content again increased significantly.

Histological studies revealed that adult eye development (the thickening of the corneagenous cells along the lateral margin of the eye) had been initiated in Stage 4 4th instar individuals. By Stage 5, these corneagenous cells, epidermal cells responsible for generating the cuticular cornea which covers the adult eye, had become more rounded. In some Stage 4 and all Stage 5 nymphs, the initiation of wing development was also observed. The orderly bilayer of columnar cells characteristic of earlier stages had vanished and the epidermal cells had begun to flatten. Thus, the deep folding of wing surfaces characteristic of later stages had always begun by Stage 5. There appears to be a gradient in apolysis and metamorphosis with these events occurring first in the head region and then proceeding posteriorly. Such an anterior to posterior gradient has been reported for other insect orders (Rembold et al., 1980; Riddiford, 1985; Gelman et al., 2000). The duration of each of Stages 4 through 7 is relatively short (less than 1 day) as compared to the other stages (Table 4), and metamorphosis (adult development) appears to occur relatively rapidly. This is especially apparent if one tracks wing development. In little more than one day, the wings have changed from simple bilayers (Stage 4) to deeply folded structures (Stage 6) (Fig. 6). Thus, by Stage 6, when the first externally visible sign of adult development is seen (diffusion of the eye spot), the wings have already expanded to near adult proportions. The cornea of the adult eye is visible by Stage 7. The exact time of apolysis and the beginning of adult cuticle deposition could not be determined from an examination of histological sections. However, ecdysteroid titer fluctuations and other histological observations (initiation of adult eye and wing development) support the view that pharate adult formation occurred prior to Stage 6, probably in Stage 4/5 4th instars.

In summary, a staging system has been designed to track development in 3rd instar and 4th instar/pharate adult greenhouse whiteflies. From a determination of soluble protein and ecdysteroid content of precisely staged 3rd and 4th instars, as well as histological examination of staged 4th instars, it appears that the molt is initiated prior to Stage 5 of the 3rd instar and at Stage 4/5 of the 4th instar. Prior to the initiation of adult development, ecdysteroid titers (fg/μg protein) were relatively high, and then, soon after, titers decreased. Studies to determine the nature of the ecdysteroids present in 4th instar/pharate adults are currently in progress. Metamorphosis, the formation of adult structures, occurs rapidly and within 1.5–2 days (by Stage 7), adult wing and eye development as well as cuticular spines were quite apparent. Our results provide important information concerning the timing and regulation of whitefly molting and metamorphosis and provide the foundation for future studies investigating the regulation as well as the inhibition of these developmental processes.

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