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Host-parasitoid interactions relating to penetration of the whitefly, *Bemisia tabaci*, by the parasitoid wasp, *Eretmocerus mundus*

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Abstract

It has been reported that the aphelinid wasp *Eertmocerus mundus* parasitizes all four nymphal instars of the sweet potato whitefly, *Bemisia tabaci* (Biotype B), with 3rd instars being the preferred hosts. The parasitoid lays its egg on the leaf underneath the host nymph. First instars hatch and later penetrate the whitefly. Previous studies have shown that the initiation of parasitoid penetration induces the host to form a cellular capsule around the parasitoid. As described here, females never oviposited once the 4th instar whitefly nymph had initiated adult development. First instar *E. mundus* larvae were observed under 2nd, 3rd and 4th instar whitefly nymphs, however, penetration did not occur until the whitefly had reached the 4th instar. The non-penetrating *E. mundus* larva almost always induced permanent developmental arrest in its 4th instar whitefly host and also caused a reduction in whole body host ecdysteroid titers. Therefore, unless there is a peak in molting hormone titer in the area local to penetration, it appears that the induction of capsule formation is not due to an increase in ecdysteroid titer. As the capsule formed around the penetrating parasitoid, host epidermal cells multiplied and became cuboidal and columnar, and relatively thick layers of new cuticle were deposited within the developing capsule, particularly near its ventral opening. The newly formed host cuticle was thinner in the dorsal part of the capsule and appeared to be absent at its apex. These results provide new information regarding the timing and dynamics of parasitoid oviposition and egg hatch as related to larval penetration, parasitoid-induced changes in whitefly development, molting hormone titers and the process of capsule formation.

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Introduction

The sweet potato whitefly, *Bemisia tabaci*, Biotype B [also known as the silverleaf whitefly (Bellows et al., 1994)] attacks more than 600 different species of plants in both field and greenhouse settings and causes billions of dollars of damage in crop losses each year. (Perkins and Bassett, 1988; Gill, 1992; Zalom et al., 1995; Heinz, 1996; Henneberry et al., 1997; HenneberryEA98; Chu and Henneberry, 1998). While chemical pesticides are the preferred method for controlling this pest (Horowitz and Ishaaya, 1995; Horowitz et al., 1994), biological control agents, especially various *Eretmocerus* (parasitoid) species have been mass reared and augmentatively released in fields in the United States and Europe to assist in the control of *B. tabaci* (Hoelmer, 1996; Roltsch et al., 2001; Simmons et al., 2002; Gould, 2003; Urbaneja and Stansly, 2004). *Eretmocerus* species have unique forms of immature development. Eggs are laid on the leaf underneath the host nymph, typically between the pairs of legs and/or near the mouthparts (Gerling et al., 1990). First instars hatch and later penetrate the host (Clausen and Berry, 1932; Gerling et al., 1990). Upon the initiation of parasitoid penetration, host epidermal cells are stimulated to undergo mitosis and eventually a capsule is formed around the parasitoid (Gerling et al., 1990; 1991). Although the function of this epidermally-derived structure is not known, it has been suggested that the capsule serves to prevent direct contact between cellular elements of the host's hemolymph and the developing parasitoid larva, which, in turn, precludes a confrontation between the parasitoid and the host's immunological systems (Gerling et al., 1990). From histological studies, it is evident that the wasp molts to the 2nd instar following the encapsulation process, whitefly tissues begin to disintegrate shortly thereafter and the capsule follows suit once the parasitoid has molted to its last (3rd) instar (Gerling et al., 1990; 1991). Parasitoid development, then, can be divided into three periods: (1) egg; (2) 1st instar larva from egg hatch until penetration, which occurs beneath the whitefly nymph; and (3) the period from the time of penetration until adult emergence which occurs within the host whitefly or its remains.

Previous studies with various *Eretmocerus* species have shown that all whitefly instars except for crawlers (i.e. the mobile, early 1st instar nymphs), are susceptible to parasitization (Gerling, 1966). However, only after a precise staging system for 4th instar development and metamorphosis to the adult stage became available (Gelman et al., 2002a; 2002b), was it possible to determine if all stages of the 4th instar and if any stages of the pharate adult are acceptable for parasitoid oviposition. Also, when an egg is laid under a 2nd as compared to a 3rd or 4th instar nymph, a longer developmental period was observed, suggesting that the parasitoid's development may be arrested when younger hosts are parasitized (Gerling, 1966). A delay in penetration and/or slower developmental rates after penetration could be responsible for the extended period of development.

There is no information concerning the timing of the early developmental process during the non-penetrating period. The influences of oviposition and of the newly hatched 1st instar larva on the host whitefly also have not been determined. Moreover, neither the timing of the processes associated with penetration nor the physiological changes that accompany penetration have been studied. Since the hatching parasitoid larva initiates these changes, they require direct host-parasitoid communication. They must involve parasitoid activity that results in the host response of epidermal cell proliferation and, ultimately, capsule formation (Gerling et al., 1990; 1991). It may be that the initiation and completion of capsule formation is associated with changes in hormone titers, for example, ecdysteroids and/or with the release of growth-influencing factors.

In the present study, we determined: (1) the latest stage of the 4th instar under which an egg will be laid; (2) the time period during which parasitoid penetration occurs; (3) the ability of the parasitoid egg and pre-penetrating 1st instar larva to induce permanent developmental arrest in its host whitefly; (4) the ability of a 1st instar parasitoid larva in the pre-penetrating and early penetrating stages to modulate host ecdysteroid titers; and (5) the morphological changes in host epidermis that occur during parasitoid penetration.

Materials and Methods

Chemicals

Twenty-hydroxyecdysone was purchased from Sigma (St. Louis, MO). The ecdysone antiserum and the peroxidase-labeled ecdysone conjugate used in the enzyme immunoassay were provided by T. Kingan (University of California at Riverside). The antiserum has a high affinity for ecdysone,

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20-hydroxyecdysone, 3-dehydroecdysone, 20,26-dihydroxyecdysone, 26-hydroxyecdysone and makisterone A (Kingan, 1989, and personal communication). Goat-antirabbit IgG and 3,3',5,5'-tetramethylbenzidine, the enzyme substrate, were purchased from Jackson Immuno Research Laboratories (www.jacksonimmuno.com) and American Qualex (www.americanqualex.com), respectively.

Insect rearing

B. tabaci were maintained in climate-controlled insect growth chambers/incubators (26 ± 2°C, photoperiod regimen 16:8 L:D and relative humidity of approximately 60%). Whiteflies were grown on different plant species including green bean, sweet potato and cotton (Gelman et al., 2002b). The sweet potatoes were planted in a potting mix as tubers until they sprouted. Issuing branches were cut and replanted. Short segments containing one to four leaves were rooted and planted in a potting mixture, and as experiments demanded, infested with whiteflies. Green beans and cotton were grown from seed and cuttings and whole plants were infested with whiteflies as needed (Gelman et al., 2002b). Whiteflies in the appropriate instar were used for experiments.

E. mundus were provided by W. Jones (SARLBCPRU, USDA, ARS, Weslaco, TX) and were maintained on whitefly-infested sweet potato plants housed in plexiglass cages having mesh-covered windows. Cages were placed in incubators (26 ± 2°C, photoperiod 16:8 L:D and relative humidity of approximately 60%). Plants having 2nd and 3rd instar *B. tabaci* were subjected to parasitization by *E. mundus*. When many parasitoids were observed to have pupated, they were placed in emergence bottles for adult collection (Gerling and Fried, 1997). For parasitization during experimental studies, leaf cages were constructed from 150 x 25 mm plastic Petri dishes. In the center of each Petri dish cover, a hole 10 cm in diameter was drilled and fine organdy mesh was secured over the opening. A slit was cut into the sidewall of the dish. Whitefly-infested sweet potato leaves were placed into the dishes, and their petioles that extended out through the slit were inserted into 12 x 75 mm capped (the cap contained small openings that were 2mm in diameter) plastic tubes that had been filled with water. Parasitoids were released into the dish at selected times.

Insect staging

Staging of 4th instar whitefly nymphs was developed by Gelman et al (2002a) as a tool for correlating developmental with hormonal changes in the greenhouse whitefly, *Trialeurodes vaporariorum* (Westwood). They described the presence of nine discernable stages of which the first five were nymphal stages and the last four were pharate adult stages. Nymphal stages were assigned based on body depth, and for adult stages on the development of the adult eye, i.e, beginning diffusion (Stage 6), light red adult eye (Stage 7), bipartite red eye (Stage 8) and dark red bipartite adult eye (Stage 9) (Gelman et al., 2002a; 2002b). This staging system was also applied to *B. tabaci,* (Gelman et al., 2002b). For this whitefly species, the molt to the adult stage occurred from either Stage 4 or 5, more commonly occurring in the former. Later it was reported that the condition of the leaf, either glaborous or pubescent influenced the maximum depth achieved by *B. tabaci* (less in the former than the latter) prior to the initiation of adult development (Gelman and Gerling, 2003). Based on molting hormone titers, it was determined that Stage-2, -3 and -4/5 nymphs reared on pubescent-leafed plants were physiologically equivalent to Stage-1, -2 young and -2 old/3, respectively, nymphs reared on glabrous-leafed plants (see Gelman and Gerling, 2003 for depth dimensions of stages). Thus, 4th instar *B. tabaci* growing on sweet potatoes pass through stages 1, 2 early, 2 late and/or 3, followed directly by stages 6, 7, 8 and 9 (Gelman and Gerling, 2003). Since many of these whiteflies pass directly from Stage 2 late to Stage 6 and since the ecdysteroid titers of Stage 2 late and 3 are not significantly different (Gelman and Gerling, 2003), for experiments described here, Stage 2 early is referred to as Stage 2 and Stages 2 late and 3 are grouped together as Stage 3.

Determination of time of penetration of the parasitoid in relation to host age

Mated *E. mundus* females were placed on sweet potato leaves with 1st and 2nd instar whiteflies, and parasitoids were allowed to oviposit for 24 h after which time they were removed. At 27° C, eggs hatched after 3 days, at which time the whiteflies had not yet reached their 4th nymphal instar so that *E. mundus* 1st instar larvae were present mostly under 3rd and occasionally under 2nd instar whitefly hosts. The condition of the hosts, whether parasitized or not, and the respective stages of the parasitoid, i.e., egg or larva, were determined by

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inverting the whitefly nymphs beginning 3 days post-parasitization, and daily for the next 2 days. Since the posterior part of a hatched larva remains in the chorion (Gerling et al., 1991), larvae were distinguished by the presence of moving mouthparts. Second, 3rd and 4th instar host nymphs with unhatched and hatched eggs were observed; many 4th instar nymphs were inverted just after the 3rd to 4th instar molt. The occurrence of parasitoid penetration was recorded.

Determination of the effects of the parasitoid egg and the non-penetrating 1st instar larva on whitefly development

In order to determine the effects of the parasitoid on the whitefly, it was necessary to follow whitefly hosts that had been parasitized by either an egg or a 1st instar *Eretmocerus* larva. For this purpose, 4th instar hosts were removed from the leaf following exposure to parasitoids, examined for parasitization, and their development was followed. In preliminary studies, it was found that when 4th instars were removed from their host plant prior to Stage 3, they did not develop to emergence. Therefore, large numbers of very young 4th instars were included among the whiteflies available for parasitization so that a sufficient number would have reached Stage 3 or higher prior to removal from the leaf. This was accomplished by infesting sweet potato leaves with whiteflies, waiting until many had reached the 3rd or 4th instar and then removing younger nymphs from the leaf. After 24 h, many of the 3rd instars had molted to the 4th instar, insuring a sufficient number of newly molted 4th instars (Stage 4-1) for the experiment. Leaves were again examined, and most of the remaining 3rd instars were removed. Older 4th instars that had initiated adult development were also present on the leaf, but were ignored for this experiment. Six -10 mated *Eretmocerus* females were introduced into leaf cages, and after 24 h at room temperature, parasitoids were aspirated from the leaves. Twenty-four or 48 h later, nymphs were inverted and the presence or absence (which served as controls) of a parasitoid was noted. For parasitized nymphs, the condition of the parasitoid, i.e., egg or 1st instar larva was recorded. All nymphs that were at Stage 6 [adult development had been initiated and the eye pigment had just begun to diffuse (Gelman et al., 2002a; 2002b)] or younger were transferred to Petri dishes containing moist paper towels and monitored every 24 - 48 hours to track development and/or adult emergence. In order to follow the developmental progress of each individual whitefly, its position on the paper towel was mapped. The percentage of whiteflies that had initiated adult development and/or had emerged was recorded. The stage of adult development that had been attained was determined by the state of the adult eye (see above).

Determination of the latest stage of the 4th instar under which a parasitoid egg will be laid

The stage of the whitefly nymphs used for determining the effects of the egg and the non-penetrating 1st instar parasitoid on the development of the host whitefly was recorded upon examination. Using information concerning the duration of each stage of the whitefly 4th instar (Gelman et al., 2002b; unpublished results), the age at which each of the nymphs had been parasitized was calculated. Thus, results of a choice experiment in which all stages between 4-1 and 4-6 and some 3rd instars (overlooked during the removal of instars younger than 4) were subjected to parasitism, were obtained. Nymphs in stages 4-8 and 4-9 were also present on the leaf at the time of parasitization; however, these were not examined for the presence of *E. mundus* eggs since previous experience had shown that eggs were only present under Stage 6 and younger nymphs (unpublished results).

Effect of 1st instar *E. mundus* in the pre-penetrating and early penetrating stages on ecdysteroid levels of 4th instar whitefly hosts

When whiteflies grown on cotton or green bean plants reached the 3rd instar, they were subjected to parasitization as described above. Three or four days later, 4th instars were inverted and those in Stage 2 that had 1st instar parasitoids in the non-penetrating stage (hatched, but clearly not attached to the whitefly i.e., not having part of the body embedded into it) or early penetrating stage (partially embedded in the host, but dislodged easily by pressing on the venter of the whitefly with a blunt object) were used for the experiment. If the parasitoid larva could not be dislodged, penetration was considered to be further along, and neither the parasitoid nor its host was used for this study. Two different samples were prepared, parasitoids alone and parasitoid and host nymph together. Since host nymphs with parasitoids removed could easily have been damaged, this group was not included in the study. Unparasitized Stage 4-2 whitefly nymphs served as controls. Material (ca. 20 individuals per

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sample) was extracted in ice-cold 75% methanol by homogenizing and sonicating the homogenate. When green bean was the host plant a waterbath-type sonicator was used (Ney Ultrasonik 300, www.blackstone-ney.com) (Gelman et al., 2002b). When cotton was the host plant a probe sonicator was used (Vibra Cell, Sonics and Materials, www.sonicsandmaterials.com) set at 40 for 20 - 30 sec. Samples were centrifuged at 4°C and 16,000 x g for 5 min. Supernatants and washes of precipitates were placed in 6 x 50 mm borosilicate glass tubes and stored in the freezer at -20° C.

An enzyme immunoassay (Kingan and Adams, 2000; as described in Gelman et al., 2002a) was used to titer the ecdysteroids in each sample. The assay's range is 500 to 40,000 fg, and results were expressed in fg 20-hydroxyecdysone equivalents/parasitoid or parasitoid whitefly combination. Ecdysteroid concentration was determined from a standard curve (semi-log plot with fg ecdysteroid plotted on the log scale) using the data analysis program "Softmax". Prior to enzyme immunoassay, tubes were dried in a Savant Speedvac Concentrator (Forma Scientific, www.forma.com).

Morphological changes in host epidermis during parasitoid penetration.

Green bean leaves infested with mostly 3rd instar whitefly nymphs were parasitized as described above. During parasitization, whiteflies were observed using a stereoscopic dissecting microscope and those believed to be parasitized were marked by placing a small dot next to the insect. 72 to 96 h later, parasitized whiteflies, and the leaf substrate upon which they rested, were fixed in Carnoy's no. 2, prepared for histological sectioning, sectioned, mounted, deparaffinized and stained (Blackburn et al., 2002). Sections were examined under a Nikon Eclipse 600 compound microscope equipped with Differential Interference Contrast optics and photomicrographs were taken using a Nikon DMX 1200 CCD camera.

Results

Determination of the latest stage of the 4th instar under which a parasitoid egg will be laid

At the temperature, humidity and photoperiodic regimens used in these studies, the duration of each of the first three *B. tabaci* 4th instar stages was approximately 24 h, and the duration of Stages 6 and 7 together was approximately 24 h.

Therefore, parasitoid larvae that were present under Stage-3, -6 or -7 4th instar nymphs or pharate adults, developed from eggs that had been deposited under 3rd instars or Stage-1 4th instars, Stage-1 or -2 4th instars and Stage-2 or -3 4th instars, respectively (Table 1). Parasitoid larvae under 4th instar nymphs at Stage 2, developed from eggs that were probably laid under 3rd instar nymphs that had been missed during the pre-infestation removal. Similarly, eggs present under Stage-6 4th instar nymphs were deposited under Stage 2 or 3, those under Stage 3 were laid under Stage 1 or 2, and those under Stage 2 were deposited under Stage-1 4th instar nymphs or 3rd instar nymphs (Table 1). Results for these experiments showed that although oviposition can occur as late as Stage 3, that occurrence is exceedingly rare. Female parasitoids never oviposited once the 4th instar nymph had undergone adult development, i.e., entered Stage 6. The stage of the 4th instar for which percent oviposition was the greatest was Stage 1.

Determination of time of penetration of the parasitoid in relation to host age

Three days after parasitization, a total of 629 1st - 4th instar whitefly nymphs were turned over, 73, 204, 146 and 206 1st, 2nd, 3rd and 4th instars, respectively, and the stage of the parasitoid (egg, non-penetrating larva or penetrating larva) was recorded (Fig. 1). At least 1 egg was found under each of the instars except the fourth, under which only 1st instar parasitoid larvae were present. Penetrating parasitoid larvae were only found under 4th instar nymphs (Fig. 1).

In other experiments, when Stage-6 and -7 pharate adult whiteflies that had been subjected to parasitization earlier in their development were turned over to determine the stage of the parasitoid, no penetration was observed in the 20 of 119 and 3 of 41 examined cases [parasitized/unparasitized 6th and 7th stage pharate adults, respectively] (Table 1). Thus, it is unlikely that parasitoid penetration occurs once the 4th instar host nymph has initiated adult development.

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Fig 1. Penetration of *E. mundus* as a function of host age. First and 2nd instar *B. tabaci* were subjected to parasitization for 24 h. Three days post-parasitization and for the next two days, 1st to 4th instar whiteflies were inverted and the occurrence of parasitoid penetration was recorded. For each whitefly instar (N1, N2, N3 and N4), bars represent the total number of unparasitized whiteflies and hosts under which a parasitoid egg, non-penetrating 1st instar or penetrating 1st instar parasitoid was present. For ease of comparison, the number within each bar represents the value of that bar and the number above each bar represents the percentage of whiteflies in a given instar that was unparasitized, or had a parasitoid egg, non-penetrating 1st instar or penetrating 1st instar parasitoid underneath them.

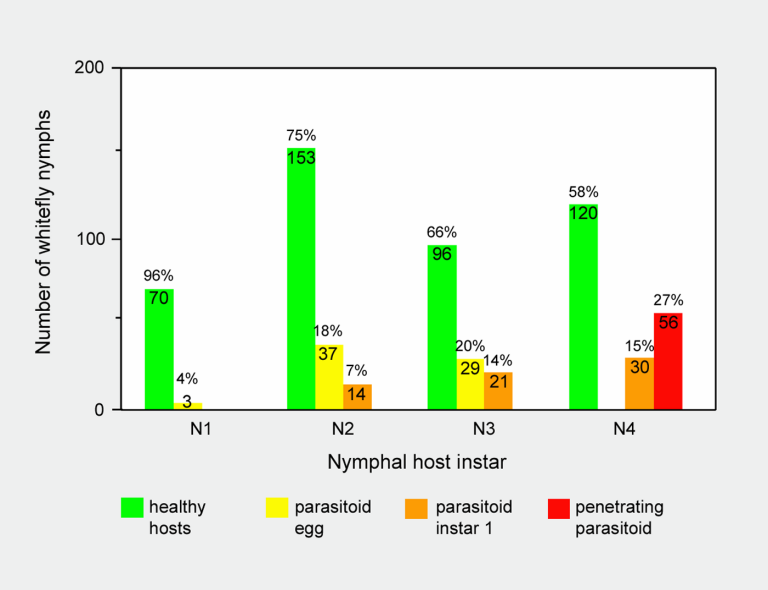


Table 1. Description of genes used for transcriptional evaluation.

Stage of 4th instar	2	3	6	7	8+
# hosts available for eggs	85	136	161	369**	-
% with parasitoid. eggs	38.8A	18.6B	2.48C	0D	-
Calculated stage of oviposition	3rd instar or 4-1	4-1 or 4- 2	4-2 or 4- 3	-	-
# hosts available for parasitoid NP larvae	131	232	119	44	334
% with parasitoid NP larvae	32.8a	33.2a	16.8b	7.3c	0d
Calculated stage of oviposition	3rd instar	3rd instar or 4-1	4-1 or 4-2	4-2 or 4-3	

Determination of the effects of the parasitoid egg and the non-penetrating first instar larva on whitefly development

When removed from the leaf, 4th instar nymphs at Stage 2, whether parasitized or not, did not develop past Stage 3. Only a small percentage progressed from Stage 2 to Stage 3, 6.0, 2.8 and 6.3%, for control, parasitized with an egg beneath the nymph (PE group) and parasitized with a non-penetrating 1st instar larva beneath the nymph (PL group) respectively (Table 2). Control, PE and PL nymphs that had attained Stage 3 of the 4th instar prior to removal exhibited 24.5, 14.8 and 0% adult emergence, respectively, and those that had reached Stage 6 of the 4th instar before being transferred to Petri dishes, exhibited 60.2, 66.7 and 6.7% adult emergence, respectively, with the 6.7% representing 1 individual (Table 2). Thus, when the parasitoid is in the egg stage, it most often does not inhibit whitefly development ending in adult emergence. However, a greater percentage of control Stage-3 and Stage-6 nymphs achieved Stage 9 (27.4 and 22.6%, respectively) than did Stage-3 and Stage-6 PE nymphs (11.1 and 0%, respectively). With a single exception, the parasitoid in the non-penetrating larval stage prevented Stage-3 and Stage-6 PL nymphs from completing development and emerging, allowing only one Stage-6 pharate adult to mature and emerge.

Effect of 1st instar *E. mundus* in the non-penetrating and early penetrating stages on ecdysteroid levels of 4th instar whitefly hosts

Whether whiteflies were grown on sweet potato (Fig. 2A) or green bean leaves (Fig. 2B) and then parasitized, both non-penetrating and penetrating *E. mundus* 1st instar larvae reduced host

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Table 2. Effect of the parasitoid egg and non-penetrating 1st instar larva on adult whitefly emergence.

Stage* of control or experimental nymphs	Number of nymphs used	Percent nymphs that reached a given stage at the termination of the experiment						
		Stage 2	3	6	7	8	9	Emerged
Control 2	50	94	6					
Control 3	106	0	39.62	4.72	0.94	2.83	27.36	24.53
Control 6	93			9.68		7.53	22.58	60.22
Exp. 2 egg	36	97.22	2.78					
Exp. 3 egg	27		62.96	11.11			11.11	14.81
Exp. 6 egg	6			33.33				66.67
Exp. 2 larvae	48	93.75	6.25					
Exp. 3 larvae	24		95.83	4.17				
Exp. 6 larvae	15			80	13.33			6.67

* Stage of control (unparasitized) and experimental (parasitized) whitefly 4th instar nymphs at the time of removal from the leaf. The stage of the parasitoid (either egg or larva) that was present underneath the whitefly nymph is indicated.

ecdysteroid titers as compared to controls (unparasitized nymphs). Mean values for the whitefly host + parasitoid (Wf + P) and for the parasitoid (P) are shown in the first two bars of Figs. 2A,B, respectively, for the non-penetrating and penetrating condition. The third bar (Wf) provides the mean value of the host alone. This mean was determined by subtracting the mean value for the parasitoid from each value for Wf + P and then determining the mean of the resultant values. Means for Wf in which the parasitoid was in the non-penetrating and penetrating condition were 38 and 48%, respectively, of the control value when whiteflies were reared on sweet potato leaves (Fig. 2A), and 20 and 17%, respectively, of the control value when the whiteflies were reared on green bean leaves (Fig. 2B). In addition, for a given plant host, a comparison of the ecdysteroid levels in the non-penetrating and penetrating parasitoids showed that the titer is significantly higher in the penetrating *E. mundus* larva.

Morphological changes in host epidermis during parasitoid penetration.

Histological techniques were used to examine parasitoid behavior prior to and during penetration (Fig. 3). Prior to penetration, parasitoid larvae were oriented with their posterior against the leaf surface, and their mouthparts against the underside of the whitefly. In the following, all orientations are with respect to the host. The pre-penetrating active *E. mundus* larva is seen with its stylets pointing towards the host while resting in the chorion until penetration (Fig. 3A). The chorion is very thin in the region surrounding the parasitoid's mouthparts. Aside from the depression created by the parasitoid, the whitefly appears to be unaffected by its presence. The host cuticle seems undisturbed, and the epidermal cells are normal in appearance.

During penetration, (Fig. 3B), the parasitoid has extended itself dorsally, pressing its mouthparts against an area of host cuticle that appears thin or absent altogether, while its posterior remains in the thickened chorion. In this particular specimen, it appears that the parasitoid is actually ingesting whitefly tissue where the host cuticle is absent. In contrast to the epidermis prior to penetration, the cellular layer at the penetration site is thicker and contains a number of rounded cells, possibly hemocytes.

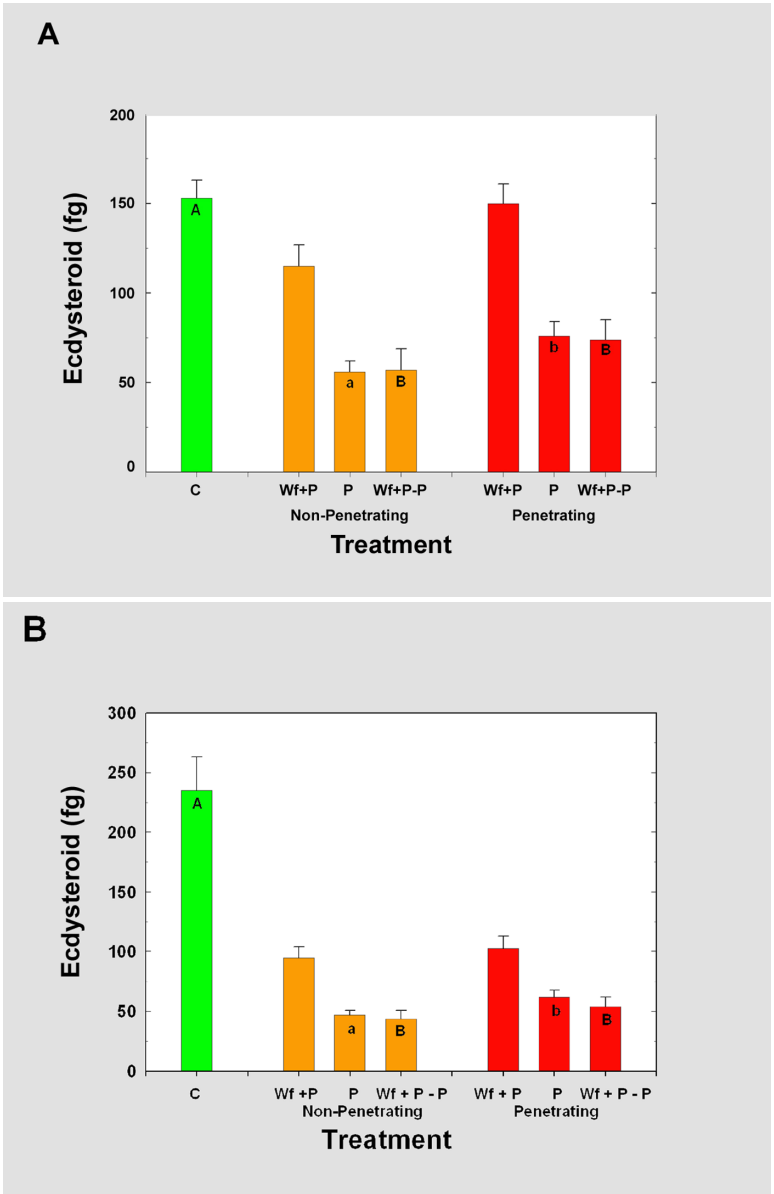
As the parasitoid continued to press against the epidermal layer and penetrate the 4th instar whitefly nymph, the host epidermal cells have multiplied and have begun to engulf the parasitoid ultimately forming a capsule around it (Fig. 3C). The cells have become distinctly cuboidal or columnar. Heavy layers of new cuticle have been deposited within the developing capsule, particularly near the opening to the outside, which is still covered by the chorion of the parasitoid egg. The newly formed host cuticle is thinner in the dorsal part of the capsule, and appears to be absent at its apex. The parasitoid larva has retracted its mouthparts at this stage, assuming the characteristic appearance of later stages.

Discussion

As a result of investigations reported here, important host-parasite interactions between *B. tabaci* and *E. mundus* have been identified and/or elucidated. These address: 1) parasitoid life cycle events that are cued by host age/stage, which, in turn, depend upon the parasitoid's ability to detect physiologically-based changes that occur during its host's development, and 2) parasitoid manipulation of host physiology and biochemistry, presumably to create an environment that is more favorable for its

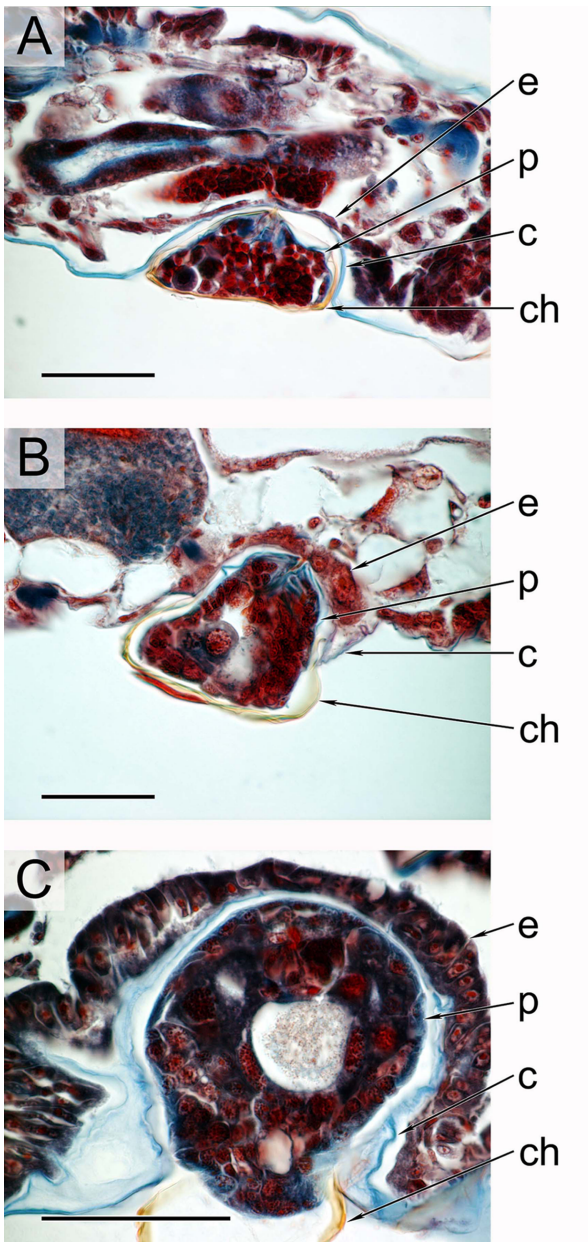
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Fig 2. Effect of *E. mundus* non-penetrating and penetrating 1st instar larvae on host ecdysteroid titers. A, whiteflies reared on sweet potato; B, reared on green bean. *B. tabaci* and *E. mundus* were reared, parasitization was performed and ecdysteroid titers were determined from whole-body methanolic extracts as described in Materials and Methods. Each value represents the mean ± S.E. of at least 11 separate determinations. C = Control Stage-2 whitefly nymphs; Wf + P= Stage-2 host whiteflies with non-penetrating or penetrating parasitoids; P = parasitoids. The third bar (Wf) for each experimental set (non-penetrating and penetrating) provides the mean value of the host alone (value obtained by subtracting the ecdysteroid titer in the sample containing parasitoids from the ecdysteroid titer in the samples containing whitefly hosts and their parasitoids). Significant difference was determined for bars having upper case letters and separately, for those having lower case letters. Bars having different lower case letters were significantly different and bars having different upper case letters were significantly different (One-way ANOVA following by Tukey's Comparsion of Means Test, $\alpha = 0.05$).



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Fig 3. Histological sections of *E. mundus* in the process of penetrating *B. tabaci*. A. *E. mundus* larva still within the chorion preparing to penetrate the whitefly nymph. The host epidermis appears normal. B. *E. mundus* larvae in the process of penetration. It is not clear whether the poorly defined mass of cells at the site of penetration are epidermal cells and/or hemocytes. C. Early capsule formation. The host epidermis is conspicuously cuboidal or columnar in appearance, and a thick cuticle is being deposited near the opening of the developing capsule. e=epidermal cells; p = parasitoid; c= cuticle; ch = chorion. Scale Bars for A-C = 50 μm.



own development. Thus: (1) when oviposition occurred under 4th instars, it was typically limited to Stages 1, 2 and 3, with relatively few eggs being laid under Stage 3. (2) Although parasitoid eggs hatched under 2nd, 3rd and 4th instar host nymphs, the larva remained under its whitefly host and did not penetrate until the whitefly had molted to its 4th instar. (3) In its pre-penetrating stage, the parasitoid larva was capable of both inducing permanent developmental arrest in its host whitefly and (4) of effecting a reduction in host ecdysteroid titers. (5) In the area of penetration, the 1st instar parasitoid larva appeared to be imbibing and/or injecting material into its host. (6) In correlation with probing by the parasitoid's mandibles, the host epidermis thickened and was abnormal in appearance. As epidermal cells multiplied in the process of capsule formation, a layer composed of cuboidal and columnar cells was generated and new cuticle was produced.

In these experiments, when mostly 4th instar whiteflies were presented for parasitization and whiteflies were inverted 48 h post-exposure, the latest host stage under which a parasitoid egg was observed was Stage 6. Since under the conditions of temperature and moisture used in these studies the maximum duration of Stages 3 and 6 together is 48 h, the latest stage under which a parasitoid egg could have been laid was a young 4th instar nymph at Stage 3. Because there were so few 6th stage nymphs under which eggs were observed, it appears that most parasitoid eggs were laid under 4th instar nymphs at Stages 1 and 2, and only a few under Stage 3. This conclusion is supported by the observation that 72 h after exposure to parasitoids, only 0.4% of 4th instar Stage-7 nymphs (3 whiteflies) were observed to have a parasitoid larva beneath them. Thus, oviposition under a 4th instar nymph at Stage 3 is relatively rare probably due to natural selection, since a whitefly in the pharate adult stage would not support parasitoid development. The rates of developmental success of *E. mundus* when attacking *B. tabaci* hosts that are in these stages have not been determined.

According to Gerling (1966), when an egg of *E. eremicus* (as *E. califonicus*) is laid under 2nd as compared to later instar greenhouse whiteflies, the developmental time period of the parasitoid is extended. Thus, it was of interest to determine whether there was also a delay in parasitoid penetration in the *E. mundus-B. tabaci* system when eggs were laid under 1st and 2nd instar hosts. In our experiments, when only these instars were offered to *E. mundus*, parasitoid eggs hatched within approximately 3 days. The duration of the 1st

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instar nymph was approximately 3 days, while the duration of both the second and the third instars of *B. tabaci* when reared on sweet potato plants at 25° C is approximately 2-3 days (unpublished results; Wang and Tsai, 1996). Thus, most of the eggs laid will hatch under 3rd and 4th instar whitefly nymphs, and relatively few should be present under 2nd instar nymphs. We observed 14, 21 and 86 *E. mundus* larvae under 2nd, 3rd and 4th instar hosts, respectively. Of the 86 larvae, 65 % were in the process of penetrating their 4th instar hosts. Since none of the 21 or 14 first instar parasitoid larvae that were observed under 2nd and 3rd instar whitefly nymphs, respectively, had initiated penetration, *E. mundus* larvae must limit their penetration to the 4th instar of the host.

Foltyn and Gerling (1985) reported that *Eretmocerus* eggs will only hatch under, and 1st instar larvae will only penetrate, a 4th instar host. While in our studies parasitoid 1st instar larvae were observed under 2nd and 3rd instar whitefly hosts, we also found that actual penetration into the whitefly host is limited to a short window, i.e., from the newly molted 4th instar until the host reaches the transformation stage into a pharate adult. This short duration permits the parasitoid to penetrate and develop within the largest and therefore most nutritious hosts, and, at the same time, to avoid having to cope with the transformation of nymphal host tissues to those of the less nutritious adult structures, i.e., wings and thicker cuticle. In order to time penetration correctly, the *Eretmocerus* female prefers to oviposit under 2nd and 3rd nymphal instars (Gerling, 1966). With an egg developmental duration of approximately 3 days at 27° C, the hatching first instar larva will typically be ready for penetration once the host has reached the 4th instar. However, invariably, some of the parasitoid eggs will hatch under 2nd or 3rd instar hosts. Thus, it was interesting to learn that when this occurs, the parasitoid larva waits for the host to molt to the 4th instar prior to penetration. The mechanism that enables this delay has not been determined. It is possible that it is host-dependent, i.e., that the host does not react to the parasitoid-induced cues for capsule formation until it is a 4th instar nymph. Alternatively, it is possible that the parasitoid, after penetrating the host's venter with its lancet-shaped mandibles is able to sense that the host has reached the 4th instar and will induce the host to initiate capsule formation. Thus, the increased duration of parasitoid development observed when oviposition occurs under younger whitefly instars is probably due, at least in part, to the delay in the initiation of parasitoid penetration. Similarly, in other host-parasite systems, e.g., *Manduca sexta* - *Cotesia congregatata* and *Heliothis virescens-Cardiochiles nigriceps*, when eggs are laid in younger instars, parasitoids remain as 1st instars until the host, *M. sexta* or *H. virescens*, molts to its last instar (Beckage and Riddiford, 1978; Pennacchio et al., 1993); then the parasitoids reinitiate development and molt to the 2nd instar. Likwise, when *E. formosa* parasitizes either *T. vaporariorum* or *B. tabaci*, the molt of the parasitoid from the 2nd to the 3rd instar is delayed until the host has initiated adult development (Hu et al., 2002; 2003). Thus, *E. mundus*, like *E. formosa*, *C. congregata* and *C. nigriceps*, delays larval development until its host is at an optimum stage/size to support parasitoid growth and metamorphosis.

The technique used to examine whether or not the presence of a parasitoid egg or non-penetrating 1st instar larva influenced the development of its host whitefly was based on the observation that some 4th instar nymphs that had developed to Stage 3 or beyond could be removed from the leaf and yet would complete adult development. Those whiteflies that had achieved Stage 6 prior to removal from the leaf had a greater success rate than those in Stage 3. This made it difficult to examine later stages, since, as discussed earlier, the number of parasitized nymphs diminished greatly once the whitefly reached Stage 3. Thus, only six Stage-6 nymphs were obtained with eggs under them, and while more replicates would have been desirable, the percent that completed development and emerged (66.6%) was very similar to the value for the unparasitized controls (60.2%), suggesting that the parasitoid egg does not induce developmental arrest in its host. However, while 22.6% of the Stage-6 controls developed to Stage 9 but did not emerge, none of the parasitized whiteflies behaved in a similar fashion. Also, the percentages of parasitized Stage-3 nymphs that reached and remained at Stage 9 (11.1%) and that emerged (14.8%) were lower than for their respective controls (27.4% and 24.5%). Considering that it does not seem plausible that some parasitoid eggs would induce permanent developmental arrest in their hosts and others would not, we cannot explain the discrepancy between control and parasitized whiteflies (eggs underneath), although it is possible that there was a disproportionate

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amount of mechanical damage to parasitized nymphs while transferring them to Petri dishes. Stage-3 and 6 test nymphs in which the parasitoid was in the pre-penetrating stage either did not develop and emerge (Stage-3 at the time of removal) or exhibited an extremely low emergence rate (Stage 6 at the time of removal). In the latter case only one of 15 nymphs (6.67 %) emerged as an adult. Thus, while the ability of the parasitoid egg to induce developmental arrest is questionable, it is clear that a non-penetrating parasitoid larva prevents adult development most of the time. Considering the fact that the E. *mundus* adult female never injures the host under which it oviposits (Gerling et al., 1999), we conclude that it is the 1st instar parasitoid larva rather than the adult female that delivers material capable of compromising the development of its whitefly host.

Both non-penetrating and penetrating *E. mundus* 1st instar larvae caused a decrease in 4th instar host ecdysteroid titers; hormone levels were between 17 and 48 % of control values. In a related homopteran, the aphid *Acyrthosiphon pisum*, ecdysteroid titers of last instar nymphs that had been parasitized as 1st instars by *Aphidius ervi* were also significantly lower than in control aphids (Pennacchio et al., 1995). There are additional reports in the literature of similar manipulations of molting hormone levels induced by both endo and ectoparasitoids for the purpose of maintaining host ecdysteroid content at levels that are most beneficial for parasitoid development (reviewed in Beckage and Gelman, 2004). Typically, parasitoids have evolved mechanisms to reduce host ecdysteroid titers and thus prevent them from molting at inopportune times, e.g., by effecting the inactivation or degeneration of host prothoracic glands, which produce ecdysone, by inhibiting the release of prothoracicotropic hormone (which stimulates the prothoracic glands to produce ecdysone) from the host brain, or by altering ecdysone metabolism so that physiologically inactive ecdysteroids are produced (reviewed in Beckage and Gelman, 2004). For ectoparasitoids, reduced ecdysteroid levels are usually evident in the host instar parasitized, and for endoparasitoids, in the host instar from which the parasitoid will emerge. Thus, in parasitized *M. sexta*, the ecdysteroid peaks that are associated with larval-pupal commitment and with the larval-pupal molt are abolished. However, a small hemolymph ecdysteroid peak was observed in the host at the time of the parasitoid's final larval molt and concomitant emergence from its host (Beckage and Riddiford, 1982; Gelman et al., 1999). Originally, since ecdysteroids have been reported to stimulate cell division in insect tissues (Oberlander, 1985; Doctor and Fristrom, 1985), we hypothesized that a parasitoid-induced increase in host ecdysteroid titers contributed to the induction of capsule formation in *B. tabaci*. Since whole body ecdysteroid titers decreased when 4th instar *B. tabaci* were in contact with non-penetrating or penetrating 1st instar *E. mundus* larvae, it appears that regulatory mechanisms that do not involve ecdysteroids are used by *E. mundus* to induce capsule formation in its host. It is also possible that a small, undetectable localized peak of ecdysteroid plays a role and/or that the reduced level of ecdysteroid is sufficient to be permissive for the action of regulatory growth factors. Thus, 20-hydroxyecdysone is essential for growth factor-induced maturation of the moth genital tract (Loeb, 1994) and for the proliferation and differentiation of lepidopteran midgut epithelial cells (Sadrud-Din et al., 1994). Also, as parasitoid penetration begins, cells that are rounded in appearance and resemble hemocytes and/or oenocytes are present in the whitefly epidermis in the area that is in contact with the penetrating parasitoid (Fig. 5b). It has been suggested that oenocytes are sites of ecdysteroid synthesis (Romer et al., 1974; Delbecque et al., 1978), but the identity of these rounded cells remains to be determined.

Our results also show that the ecdysteroid level of penetrating parasitoids (whiteflies raised on both sweet potato and green bean, Fig 2A and B, respectively) was significantly higher than in non-penetrating parasitoids. This is probably because penetrating *E. mundus* 1st instar larvae were preparing to molt to the 2nd instar, an event that occurs during or at the end of the encapsulation process (Gerling et al., 1990; 1991).

Although the manner in which capsule formation is controlled in the *B. tabaci* - *E. mundus* system remains unknown, our results build on the model proposed by Gerling et al. (1990; 1991) to describe its formation. Based on observations of histological sections, these authors reported that the *E. mundus* larva pierces the venter of its host, is surrounded by a capsule that is generated by its host and in the process, enters the host. The cells of the host epidermis that are located just above the larva grow and multiply and the entrance to the capsule, but not the interior region above the parasitoid's head,

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is covered with cuticle. Our results support this view and show the parasitoid stylets of a pre-penetrating larva in the process of piercing the host whitefly and of imbibing from and/or injecting material into the whitefly. It is likely that when the parasitoid probes the nymph with its stylets it can determine the developmental state of the host, and since, as mentioned earlier, a non-penetrating parasitoid almost always induces permanent developmental arrest in its host, the parasitoid could be injecting a material that prevents the whitefly from completing development. The abnormal appearance of the host epidermis as the *E. mundus* larva begins to penetrate is noteworthy. The identity and function of the rounded cells among the epidermal cells (e) of Fig. 3B is not known, but they could be hemocytes or, perhaps, oenocytes that have been reported to be present in insect epidermis (Wigglesworth, 1976). As penetration continues, epidermal cells multiply and are cuboidal or columnar in appearance rather than flattened as in the normal epidermis. The advantage of a capsule composed of cuboidal and/or columnar cells as opposed to flattened epidermal cells is not known. Since once penetration is completed, the cuticle lining the capsule disappears (Gerling et al., 1990), it may be that the parasitoid is capable of digesting the host-produced cuticle and that digestion proceeds more rapidly in the interior portion of the capsule, i.e., dorsal to the parasitoid.

In conclusion, our studies serve to complement both the scientific information concerning the commercially important genus *Eretmocerus,* information that will, hopefully facilitate its mass rearing and use in pest management. The unique mode of parasitization, from host penetration to host consumption, raises questions that differ from those asked with other whitefly parasitoids, such as the interdependence or communication between the hatching parasitoid larva and the host. These can be broken down to information about the limits of host ages suitable for parasitoid penetration, the success of the parasitoid in recognizing these limits, and the influence that the parasitoid stages have upon the host prior to and during actual penetration. Answers to these questions can open the way both to scientific research concerning the mode of communication between host and parasitoid and to practical technology of optimizing the time of parasitization for biological control of whiteflies.

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