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Identification of the molting hormone of the sweet potato (*Bemisia tabaci*) and greenhouse (*Trialeurodes vaporariorum*) whitefly

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Abstract

In order to identify the whitefly molting hormone, whole body extracts of mature 4th instar and newly formed pharate adult *Bemisia tabaci* (Biotype B) and *Trialeurodes vaporariorum* were prepared and subjected to reverse phase high performance liquid chromatography (RPHPLC). Ecdysteroid content of fractions was determined by enzymeimmunoassay (EIA). The only detectable ecdysteroids that were present in significant amounts in whitefly extracts were ecdysone and 20-hydroxyecdysone. The concentrations of 20-hydroxyecdysone in *B. tabaci* and *T. vaporariorum* extracts, respectively, were 40 and 15 times greater than the concentrations of ecdysone. The identity of the two ecdysteroids was confirmed by normal phase high performance liquid chromatography (NPHPLC). When ecdysteroid content of RPHPLC fractions was assayed by radioimmunoassay (RIA), small amounts of polar ecdysteroids were also detected indicating that these ecdysteroids have a very low affinity for the antiserum used in the EIA. Ecdysteroid at 10.4 mM administered by feeding stimulated 2nd instar whitefly nymphs to molt. Based on our results, it appears that 20-hydroxyecdysone is the whitefly molting hormone.
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Keywords: Whitefly; *Bemisia tabaci* (Biotype B); *Trialeurodes vaporariorum*; Molting hormone; 20-hydroxyecdysone; Ecdysteroids

1. Introduction

The sweet potato whitefly (SPWF), *Bemisia tabaci*, Biotype B (also known as the silverleaf whitefly (Bellows et al., 1994)) is polyphagous, attacking more than 600 different species of plants in both field and greenhouse settings, including food, fiber and ornamental species. The greenhouse whitefly (GHWF), *Trialeurodes vaporariorum* is also a polyphagous species and is a serious pest of plants grown in greenhouses. World-wide, these homopterans cause billions of dollars of damage in crop losses each year by feeding on plant phloem, transmitting plant pathogenic viruses (primarily *B. tabaci*) and producing honeydew that causes stickiness and supports the growth of sooty mold (Perkins and Bassett, 1988; Gill, 1992; Zalom et al., 1995; Heinz, 1996; Henneberry et al., 1997, 1998; Chu and Henneberry, 1998). Precise staging systems for identifying both instar and for tracking developmental progress of 3rd instar and of 4th instar/pharate adult whiteflies grown on green bean plants have been described (Gelman et al., 2002a, b). Since it was later found that leaf pubescence affects the maximum depth achieved by 4th instar/pharate adult *B. tabaci*, the system was modified for 4th instar *B. tabaci* grown on glabrous-leaved plants (Gelman and Gerling, 2003). The development of a precise staging system for 4th instar and pharate adult whiteflies made it possible to monitor ecdysteroid (the molting hormone, its precursors and metabolites) titer fluctuations during these stages and to determine the timing and magnitude of the premolt peak in 4th instar nymphs. Although peak titers were very low, <2.0 pg/whitefly (Gelman et al., 2002a, b), since the identity of the whitefly molting hormone is unknown, we undertook to use high

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performance liquid chromatography (HPLC) coupled with enzymeimmunoassay (EIA) of HPLC fractions to identify the ecdysteroids present in the nymphal-adult ecdysteroid peak of both the GHWF and SPWF.

2. Materials and methods

2.1. Chemicals

The ecdysone antiserum and the peroxidase-labeled ecdysone conjugate used in the EIA were provided by T. Kingan (University of California at Riverside, USA). The antiserum has a high affinity for ecdysone (E), 20-hydroxyecdysone (20E), makisterone A, 20,26-dihydroxyecdysone (20,26E), 26-hydroxyecdysone (26E) and 3-dehydroecdysone (Kingan, 1989; Kingan, pers. Commun.). The 3,3′,5,5′-tetramethylbenzidine (TMB, enzyme substrate) and the goat antirabbit IGG were obtained from American Qualex (San Clemente, CA, USA) and from Jackson Immuno Research Laboratories (West Grove, PA, USA), respectively. Both E and 20E were purchased from Sigma (St. Louis, MO, USA), and 26E and 20,26E were gifts of M. J. Thompson, (formerly of the Insect Physiology Laboratory, USDA, ARS, Beltsville, MD). Tritiated ecdysone (63.5 Ci/mmol) used in the radioimmunoassay (RIA) was purchased from New England Nuclear Corp. (Boston MA, USA). The ecdysone antiserum used in the RIA was prepared by W.S. Bollenbacher (University of North Carolina, Chapel Hill, NC, USA) from a hemisuccinate derivative of ecdysone (at the C-22 hydroxyl group) that had been coupled to thyroglobulin. It has a high affinity for E, 20E, 26E, 20,26E and makisterone A.

2.2. Insect rearing

Whiteflies were reared on a variety of plants, including green bean cv. Roma II (Burpee, Warminster, PA, USA), sweet potato, tomato cv. Bush Big Boy (Burpee, Warminster, PA, USA), cotton cv. Stoneville ST 474 (Stoneville Pedigreed Seed Co., Maricopa, AZ, USA), collard cv. Champion (Meyer Seed Co., Baltimore, MD, USA) poinsettia cv. Freedom Red (Paul Ecke Ranch, Encinitas, CA, USA) and eggplant cv. Millionaire Hybrid (Burpee, Warminster, PA, USA). Sweet potato was propagated vegetatively from the sweet potato tuber (purchased at the local supermarket) and poinsettia was grown from cuttings supplied by Paul Ecke Ranch (Encinitas, CA, USA). All other plants were grown from seed. Plants were fertilized weekly using a 1% solution of Mira-Gro (15% N, 30% P_2O_5, 15% K_2O) (Miracle-Gro Products, Inc., Port Washington, NY). The SPWF colony was maintained in a walk-in, climate-controlled insect growth chamber (26 ± 2 °C, L:D 16:8 and relative humidity of 60–80%), while the GHWF colony was maintained in a greenhouse under similar environmental conditions. Green bean plants were infested by placing greenhouse-grown plants into the growth chamber that housed our SPWF colony or into the GHWF colony. Leaves of colony plants were held above the new plants and were shaken vigorously so that whiteflies would move onto the new plants. After 6 h, adult whiteflies were removed from the newly infested plants, and these plants were transferred to a second growth chamber or to an incubator box and maintained under the same rearing conditions as described previously for the colonies. Plants infested with GHWF and SPWF were maintained in separate chambers.

2.3. Insect staging

GHWF and SPWF 4th instar/pharate adults were staged as described elsewhere (Gelman et al., 2002a, b). Briefly, Stages 1, 2 and 3 were characterized by body depths of 0.1, 0.15, 0.2 ± 0.02 mm, respectively; stage 4 had a body depth of 0.23–0.26 mm. Nymphs with a body depth $\geqslant 0.27$ mm were assigned to Stage 5. Stages 6–9 were identified based on the appearance of the developing adult eye. Nymphs entered Stage 6 when the small intense red dot characteristic of the eye of Stages 1–5 began to diffuse. Stages 7–9 were characterized by a light red, medium red bipartite, and dark red or red-black bipartite adult eye, respectively. An optical micrometer mounted on a stereoscopic microscope was used to identify 4th instar nymphs (0.6–0.8 mm in length) and to identify stage 4/5 GHWFs and SPWFs. Whiteflies from more than one hundred different plants were sampled for each species of whitefly.

2.4. Generation of ecdysteroid profiles using reverse phase high performance liquid chromatography (RPHPLC) followed by EIA

Since ecdysteroid titers peak in Stage-4/5 4th instar GHWFs and Stage 4-Early 6 SPWFs (Gelman et al., 2002a, b), these whiteflies were removed from host plants in groups of 20 and placed in 1.5 ml microcentrifuge tubes each containing 75 µl of ice cold 75% aqueous methanol. Tubes containing either GHWFs or SPWFs were stored at −20 °C prior to ecdysteroid extraction. Whiteflies were homogenized, homogenates were sonicated and centrifuged as described in Gelman et al. (2002a), and supernatants plus washes were combined and placed in 10×75 mm borosilicate glass tubes (approximately 1.5 ml per tube) that were sealed with parafilm and stored at −20 °C prior to RPHPLC. Tubes were dried in a Savant Speedvac Concentrator (Forma Scientific, Marietta, OH, USA) and contents (extracts of 1000–1500 whiteflies) were combined in 60 µl

of 40% methanol for injection onto the column. RPHPLC was performed using a Luna C_{18} column (3.0 mm i.d. × 250 mm column, 5 μm particle size), on a Hewlett Packard 1100 HPLC. The solvent was 40% methanol, the flow rate was 350 μl/min, the column temperature was 31 °C and 1 min fractions were collected. E and 20E standards were injected before or after each RPHPLC sample run to correlate elution times of standards with those of fractions. Between runs, the injector was washed exhaustively with the eluting solvent and the column was washed with 50% methanol–50% isopropanol (5 × 1 ml injections) followed by equilibration with 50 column volumes of 40% methanol to insure that any lingering ecdysteroid from a prior run was eluted. Elution times of ecdysteroid standards were determined prior to or after each sample run. Following RPHPLC on the first set of standards, the column was washed as described above, a blank injection of 40% methanol was performed, and fractions were dried and assayed for ecdysteroid. No ecdysteroid was detected in any of the fractions demonstrating that there was no carry over of ecdysteroid from a prior run.

The amount of detectable ecdysteroid that was present in an aliquot of each fraction was determined by using an EIA (range = 500–40,000 fg) developed by T. Kingan (Kingan and Adams, 2000; Gelman et al., 2002a). Aliquots of HPLC fractions were dried in the Speedvac Concentrator and reconstituted in 50 μl of EIA buffer prior to assay. The EIA is performed in a 96-well microtiter plate and is based upon the competition between ecdysteroid (in standard or sample) and a known amount of peroxidase-labeled conjugated ecdysone for the ecdysteroid antiserum that has been bound to the IGG-coated wells. After several washes, the addition of TMB substrate (developed a blue color) followed by phosphoric acid (1 M) produced a yellow color. Absorbance was measured at 450 nm. Ecdysteroid present in each sample was determined from a standard curve (semi-log plot with fg ecdysteroid plotted on the log scale). Corrections were made for percentage loss (typically 10–25%) by comparing the sum of ecdysteroid activity in the fractions with the amount of ecdysteroid present in the original sample before HPLC and multiplying the ecdysteroid content of each fraction by the appropriate correction factor. The identity of ecdysteroids in active fractions was determined from the elution profiles of ecdysteroid standards.

In order to determine the ability of the EIA antiserum to detect polar ecdysteroids that, under the conditions described above, typically elute in RPHPLC fractions 5–8 (Gelman and Woods, 1986; Gelman et al., 1988) when RIA is used to assay ecdysteroid, a whole-body extract prepared from Stage-4-Early 6 4th instar sweet potato whiteflies was subjected to RPHPLC as described above. Sixty microliters of extract prepared from approximately 1000 SPWFs were injected onto the column. Aliquots of RPHPLC fractions were subjected to EIA (as described above). The remaining eluant in each fraction was also assayed for ecdysteroid using RIA (Gelman et al., 1997) rather than EIA.

2.5. Confirmation of ecdysteroid identity using normal phase high pressure liquid chromatography (NPHPLC)

To verify the identity of ecdysteroids that eluted in RPHPLC fractions that showed activity in the EIA, the remainders of fractions from a C_{18} column run that composed a given peak were combined and subjected to a second fractionation on a Shandon Hypersil silica normal phase column (4.6 mm i.d. × 25 cm, 5 μm particle size) with 125 parts methylene chloride, 35 parts isopropyl alcohol and 2.5 parts water as the eluting solvent. The flow rate was 1.0 ml/min, the column temperature was 23 °C, and 1.0 min fractions were collected. Ecdysone and 20E standards were injected before or after each sample normal phase run to correlate elution times of standards with those of fractions. Between runs, the injector was washed exhaustively with the eluting solvent including 5 × 1 ml injections of the eluting solvent and then the column was equilibrated with 50 column volumes of the eluting solvent.

2.6. Administration of 20E to sweet potato whitefly nymphs reared on artificial diet

SPWFs were reared under sterile conditions as described by Jancovich et al. (1997). Briefly, 6-day-old whitefly eggs (1 day prior to hatch) were collected from heavily infested green bean leaves, surface sterilized and placed on membranes (MSI Tef Sep Teflon disc filters, Fisher Scientific, Pittsburg, PA) positioned between the chambers that contained SPWFs and diet. Whiteflies were fed an aqueous diet containing 15% sucrose and 5% yeast extract. Diets were passed through 0.22 μm filters prior to addition to the feeding chambers. Eight to 10 days after hatch, membranes were examined and the positions of young 2nd and young 3rd instar nymphs were mapped. A 250 μl aliquot of fresh diet containing sufficient 20E to generate final concentrations in the feeding chambers of 0.021, 0.21, 2.1 and 10.4 mM 20E was injected into the diet chambers. Control chambers received 250 μl of diet without the added 20E. There were at least three replicates for each concentration of 20E tested. Whiteflies were observed 24 h later, and the number of whiteflies that had molted from the 2nd to the 3rd and the 3rd to the 4th instar was recorded. A jump in whitefly length (Gelman et al., 2002a, b) and the presence of an exuvium were the characteristics used to signify that a molt had occurred. For each feeding

chamber, percent molt for each treatment for each instar (2nd and/or 3rd) was calculated. A one-way ANOVA followed by the Fisher's least significant difference (LSD) comparison of means test was used to determine if mean percent molt on the day following the administration of 20E was significantly different for experimental and control whiteflies ($\alpha = 0.05$).

3. Results

3.1. RPHPLC profiles of extracts of sweet potato whitefly and greenhouse whitefly

RPHPLC/EIA ecdysteroid profiles of whole-body extracts of 4th instar Stage-4-Early 6 SPWFs and Stage 4/5 GHWFs are shown in Fig. 1A and B, respectively. Results of duplicate runs were similar. Ecdysteroids that have the same elution times as 20E and E standards were present in both whitefly extracts. No other antiserum-detectable ecdysteroids were present in significant quantities. At the time of sampling there was considerably more 20E than E in whitefly extracts. After correcting for the relative affinity of the antiserum for E and 20E (2:1) for the three RPHPLC runs for SPWF and the two RPHPLC runs for greenhouse whitefly, there were means of 40 and 15 times more 20E than E, respectively.

RPHPLC/EIA and RPHPLC/RIA ecdysteroid profiles of whole body extracts of Stage-4-Early 6 SPWF are compared in Fig. 2. While polar ecdysteroids were detected by the antiserum used in the RIA (in fractions 6–8 and 15), the EIA antiserum was unable to detect significant quantities of these ecdysteroids. Otherwise, the profiles generated using the RIA and EIA were similar. The irregularities in the E peak generated by the RIA are due to the low levels of E present, levels that are below the 50 pg lower limit of the RIA. Since the relative affinities of the ecdysteroids composing the polar ecdysteroid peak are not known and since the amounts of ecdysteroid present in these peaks are at the lowest level of reliable detection of the RIA, a comparison of the relative amounts of polar ecdysteroid with E or 20E was not possible.

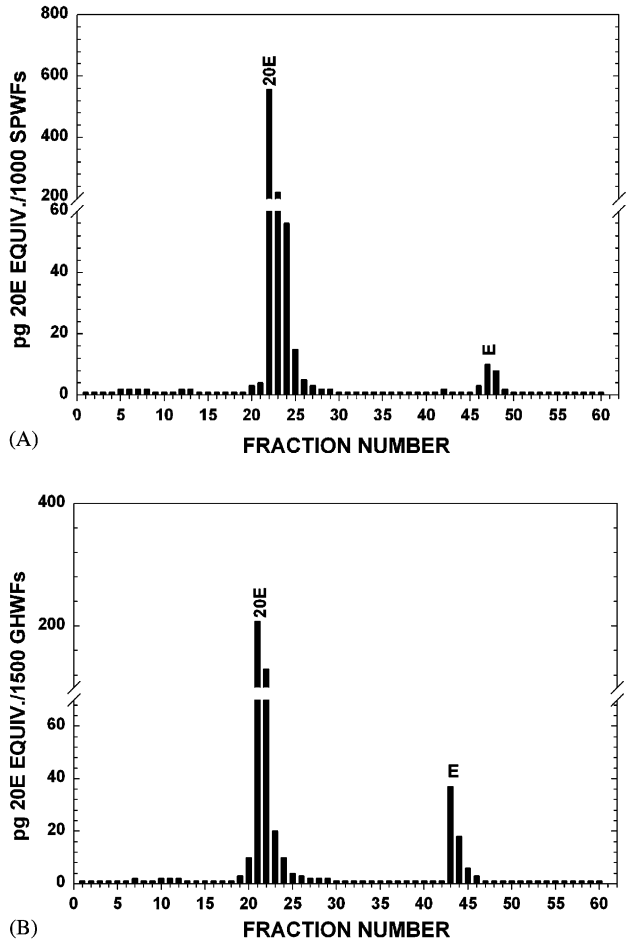

Fig. 1. Ecdysteroid profiles of extracts of Stage-4 through Early 6 SPWF (A) and of Stage 4/5 GHWF (B) generated by RPHPLC/EIA. Extracts were prepared and injected onto a RPHPLC Luna C_{18} column (3.0 mm i.d. × 250 mm column, 5: particle size), on a Hewlett Packard 1100 HPLC as described in Section 2. The solvent was 40% methanol, the flow rate was 350 μl/min, the column temperature was 31 °C and 1 min fractions were collected. Fractions were assayed for ecdysteroid content by EIA. Elution times of E and 20E standards are indicated. Values for fractions making up the E peak were not corrected for the greater relative affinity of the antiserum for ecdysone. Results are for a single RPHPLC run; similar profiles were generated from duplicate runs.

3.2. Confirmation of ecdysteroid identity using NPHPLC

From selected RPHPLC runs, the remaining volume of fractions having the same elution time as 20E or E were combined individually and processed for NPHPLC. For both ecdysteroids, EIA-positive fractions had the same elution times as their respective ecdysteroid standards. Results for the SPWFs are illustrated in Figs. 3A and B. Results for the GHWFs were similar with peak 20E activity eluting in Fraction 12 and peak E activity eluting in Fraction 10 (results not shown).

3.3. Effect of 20E on instar duration of sweet potato whitefly nymphs reared on artificial diet

At doses of 0.21–2.1 mM, 20E did not significantly affect the percent molt of 2nd or 3rd instars as compared to control SPWF nymphs (Table 1). However at a dose of 10.4 mM, there was a significant increase in percent molt from the 2nd to the 3rd, but not the 3rd to the 4th instar. Thus, oral administration of 20E, at the highest

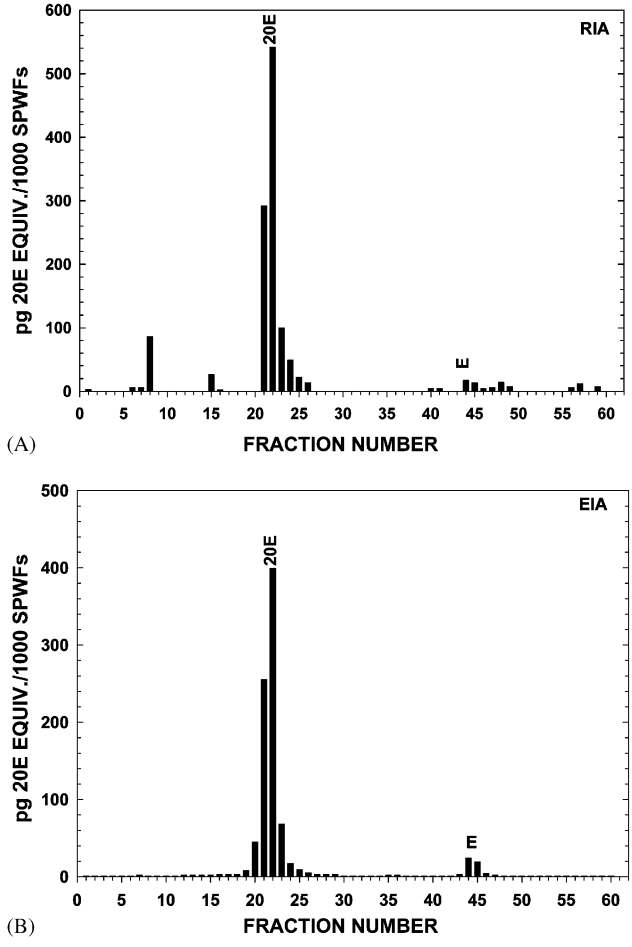

Fig. 2. Ecdysteroid profile of an extract of Stage-4 through Early 6 sweet potato whitefly (SPWF) generated by RPHPLC/RIA (A) and RPHPLC/EIA (B). Information regarding extract preparation and RPHPLC are described in the legend to Fig. 1 and in Section 2. Fractions were assayed for ecdysteroid content by RIA and EIA. Elution times of E and 20E standards are indicated.

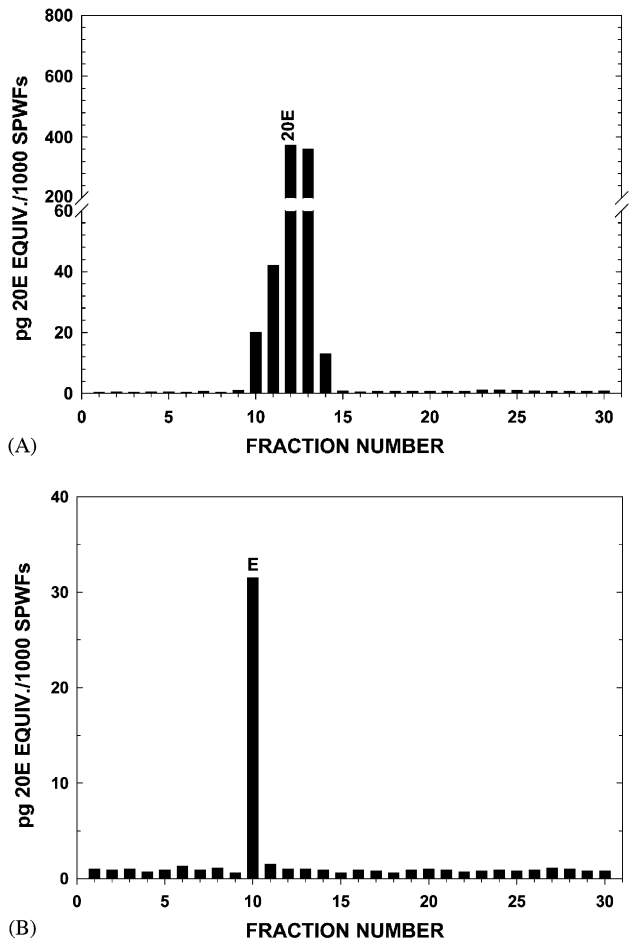

Fig. 3. NPHPLC confirmation of RPHPLC peaks having the same elution times as 20E (A) and E (B). RPHPLC fractions associated with 20E and E peaks were combined individually and processed for injection onto a NPHPLC Shandon Hypersil silica gel column (4.6 mm i.d. × 25 cm, 5: particle size) with 125 parts methylene chloride, 35 parts isopropyl alcohol and 2.5 parts water as the eluting solvent. The flow rate was 1.0 ml/min, the column temperature was 23 °C, and 1.0 min fractions were collected (see Section 2). Fractions were assayed for ecdysteroid content by EIA. Elution times of E and 20E are indicated. Results for both 20E and E are for a single NPHPLC run; similar profiles were generated from duplicate runs. SPWF = sweet potato whitefly.

dose of 20E tested, only induced 2nd instar whitefly nymphs to molt.

4. Discussion

Twenty hydroxy ecdysone was the major ecdysteroid detected in extracts of SPWF and GHWF at the time of the nymphal–adult molt, and when administered in the diet at a dose of 5 μg/μl (final concentration in the diet = 10.4 mM), 20E induced 2nd instar SPWF nymphs to molt. These results strongly suggest that this ecdysteroid is the molting hormone of whiteflies and other homopterans. Twenty-hydroxyecdysone has been reported to be the molting hormone of most insect species examined, insects that are primarily in the orders, Lepidoptera, Orthoptera, Diptera and Hymenoptera (Riddiford and Truman, 1978; Kelly et al., 1981; Koolman and Karlson, 1985). In general, phytophagous insects use dietary sterols to produce cholesterol that, in turn, is converted via a complex metabolic pathway to E, the immediate precursor of 20E (Koolman, 1980; Svoboda and Thompson, 1983; Rees, 1985). However, for some hemipteran (e.g., *Oncopeltus fasciatus*, the milkweed bug) and hymenopteran (e.g., *Apis mellifera*, the honey bee) insects that cannot convert C_{28} and C_{29} sterols to C_{27} sterols via dealkylation at the C-24 position (i.e., cholesterol and its metabolites), makisterone A, a C_{28} ecdysteroid, serves as

Table 1
Effect of 20E on percent molt of diet-reared sweet potato whitefly 2nd and 3rd instars 24 h post-administration of 20E

	Dose of 20E (mM)				
	0 (Control)	0.021	0.21	2.1	10.4
Molt					
2nd → 3rd	13.9 ± 3^{a}	9.3 ± 2^{a}	14.4 ± 5^{a}	3.0 ± 0.2^{a}	55.0 ± 13^{b}
3rd → 4th	16.1 ± 3^{A}	16.8 ± 3^{A}	3.5 ± 2^{A}	3.1 ± 1^{A}	21.2 ± 10^{A}

The positions of 2nd and/or 3rd nymphal instars were mapped 8–10 days after mature eggs were transferred to the membranes of diet chambers. Sufficient 20E was added to the diet on day 0 to yield the final test concentrations indicated. Each value represents the mean ± S.E. of at least 3 separate determinations. A one-way ANOVA followed by the Fisher's least significant difference comparison of means test was used to determine if the mean percent molt on the day following the administration of 20E was significantly different for experimental and control whiteflies ($\alpha = 0.05$). For each instar, means with the same letter designation were not significantly different.

the molting hormone (Kaplanis et al., 1975, 1980; Kelly et al., 1981; Svoboda et al., 1983; Kelly et al., 1985; Feldlaufer et al., 1985). RPHPLC/EIA or /RIA of whitefly extracts (this study) did not reveal an ecdysteroid peak that had the same elution time as makisterone A; nor were significant amounts of any other ecdysteroids detectable by the antisera used in the EIA and RIA, observed, except for some polar ecdysteroids (probably metabolic deactivation products of 20E and E (Koolman, 1980; Rees, 1985; Koolman and Karlson, 1985)) that were detected by the antiserum used in the RIA. Since 20E rather than makisterone A is the major SPWF and GHWF ecdysteroid detected at the time of the molt, it appears that these whiteflies and probably other homopterans are able to convert C_{28} sterols to cholesterol.

Typically, insects in which 20E serves as the molting hormone, when injected with 20E, will exhibit a molt. Since it would have been very difficult to inject a whitefly due to its small size, we administered ecdysteroid by feeding whiteflies on an artificial diet that contained 20E at doses of between 0.021–10.4 mM. Only at the highest dose did 20E stimulate molting in 2nd instar SPWFs, but this dose was not sufficient to stimulate 3rd instars to molt. The administration of E or 20E via feeding rather than directly into the hemolymph is often not as effective due to metabolism in the gut followed by excretion of metabolites (Zhang and Kubo, 1993; Blackford and Dinan, 1997). Thus, while many species of insects exhibit abnormalities in growth and development when fed on diets containing 20E, polyphagous lepidopterous insects are able to tolerate a relatively high concentration of 20E in their diets without showing any adverse effects (Blackford et al., 1996; Robinson et al., 1987; Zhang and Kubo, 1993). Since the SPWF is exceptionally polyphagous, it is possible that the 20E imbibed by the this whitefly was similarly metabolized in the gut and converted to physiologically inactive ecdysteroids, thus making this whitefly relatively resistant to the effects of the 20E.

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