Larval Development of *Diaphorencyrtus aligarhensis* (Hymenoptera: Encyrtidae), an Endoparasitoid of *Diaphorina citri* (Hemiptera: Psyllidae)

Eric Rohrig,[1,2,3] Paul D. Shirk,[2] David G. Hall,[4] and Philip A. Stansly[5]

ABSTRACT   The koinobiont endoparasitoid *Diaphorencyrtus aligarhensis* (Shafee, Alam & Agarwal) (Hymenoptera: Encyrtidae) is an imported biological control agent being released in Florida against the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae). The eggs and early larvae were found free floating within the hemocoel. Larvae were soft bodied with no observable hairs, bristles, or external appendages, such as anal vesicles, in any instar. By the third instar, larvae had begun attaching to nymphal tissues by anal secretions that provided a means of orienting within the host nymph. The penultimate and last-instar larvae were found with their posterior anchored in the head–thoracic region of the host with their head oriented toward the posterior of the host nymph. Before the beginning of the prepupal stage, the host nymph was turned into a mummy and glued to a plant surface, apparently requiring some secretions from the wasp larva. Development from oviposition to adult eclosion of *D. aligarhensis* took ≈16 d at 25°C when oviposition occurred in second- through early fourth-instar nymphs, although this time was shortened by 4 d (25%) when the wasps oviposited in mid-fourth-instar *D. citri* nymphs. This reduction in developmental time did not affect wasp fertility or life span and may offer a significant approach to improved rearing of this wasp for augmentative releases to control the Asian citrus psyllid.

KEY WORDS   Asian citrus psyllid, Huanglongbing, citrus greening disease, anal secretions

The Asian citrus psyllid, *Diaphorina citri* Kuyawama (Hemiptera: Psyllidae), is a serious pest of citrus (*Citrus* spp.) and its close relatives (McClean and Schwarz 1970). Immature *D. citri* feed on sap from the phloem of young soft shoots and buds, causing leaf damage and distortion or death of new shoots (Mead 1977). In addition, psyllids secrete large quantities of honey-dew, promoting the growth of sooty mold that can inhibit photosynthesis as well as tarnish fruit appearance (Wang et al. 2001). However, damage from direct feeding is of little consequence compared with the transmission of the phloem-limited gram-negative bacterium *Candidatus* Liberibacter asiaticus Jagoueix, Bové and Garnier (α-Proteobacteria) (Jagoueix et al. 1994) that results in a systemic tree infection leading to poor fruit production and tree decline (McClean

and Schwarz 1970). This disease is commonly referred to as citrus greening due to the characteristic of affected fruit to remain green at the peduncular end (Bove 2006). The disease is also known as huanglongbing (HLB), Chinese for "yellow shoot disease" in reference to the initial foliar symptoms (van Vuuren 1996). Greening was first reported in Miami-Dade County, FL, in September 2005 and has since been confirmed in >30 Florida counties (Morris and Muraro 2008) as well as Georgia, South Carolina, and Louisiana (NAPPO 2008, 2009a,b).

Two host specific solitary koinobiont parasitic wasps, *Tamarixia radiata* (Waterston) and *Diaphorencyrtus aligarhensis* (Shafee, Alam & Argarwal) (Hymenoptera: Encyrtidae), were imported from Taiwan into Florida in 1998 (Hoy and Nguyen 1998) and released into Florida beginning in 1999 as biological control agents for *D. citri*. *T. radiata* was released in a successful effort to reduce psyllid populations in Réunion Island (Étienne and Aubert 1980) and both parasitoid species have been attributed with a significant role in biological control of the psyllid in Taiwan (Chien and Chu 1996). Currently, *T. radiata* has established in many regions of Florida, providing varying levels of control (Qureshi et al. 2009), whereas *D. aligarhensis* has not yet established.

In September 2006, an additional population of *D. aligarhensis* was collected in Guangdong province,

[1] Department of Entomology and Nematology, University of Florida, Gainesville, FL 32611.

[2] USDA–ARS Center for Medical, Agricultural and Veterinary Entomology, Gainesville, FL 32608.

[3] Corresponding author, e-mail: eric.rohrig@ars.usda.gov.

[4] USDA–ARS Horticultural Research Laboratory, Fort Pierce, FL 34945.

[5] University of Florida-IFAS, SWFREC, Immokalee, FL 34142.

China (R. Nguyen, personal communication), and sent to Florida where a colony was initiated in the Maximum Security Room of the Florida Biological Control Laboratory, Florida Department of Agriculture and Consumer Services, Division of Plant Industry, Gainesville, FL. Voucher specimens were deposited in the Florida State Collection of Arthropods, Gainesville, FL. Parasitoids were held in quarantine through 20 generations before release permitting was complete. The goal of this importation from a different region of China was to rear, study, mass release, and establish this population of *D. aligarhensis* that may better adapt and compete in Florida as a biological control agent of *D. citri.*

Polymerase chain reaction (PCR) analysis proved the 1998 Taiwan population of *D. aligarhensis* to be positive for *Wolbachia* (Jeyaprakash and Hoy 2000). When the same PCR analysis procedure was repeated using this mainland China wasp population, a positive result was obtained (E.R., unpublished data). Infection with *Wolbachia* is the most likely cause of thelytoky in these wasps (Stouthamer et al. 2002, Hagimori et al. 2006, Meyer and Hoy 2007). *D. aligarhensis* wasps reportedly use second–fourth instars for oviposition and host feed on first–fourth instars (Skelley and Hoy 2004). However to date, there has been no published study on larval development of *D. aligarhensis.* This report establishes a developmental profile for *D. aligarhensis* embryos and larvae oviposited in second-, third-, and fourth-instar nymphs of *D. citri.* This information pertinent to the development of this wasp provides a foundation to improve culturing for augmentative releases as well as add to the general knowledge of *D. aligarhensis* and *D. citri* biology.

Materials and Methods

Insect Colonies. *D. citri* colonies were maintained on orange jasmine, *Murraya paniculata* plants in 3.78-liter pots with a 30:70 mix of vermiculite (Jungle Growth Professional Growers Mix potting soil, Piedmont Pacific, Inc. Statham GA). Plants were maintained under natural sunlight in a greenhouse in Gainesville, FL. Temperatures were maintained between 27 and 32°C and the relative humidity varied from 40 to 70%. Freshly flushing plants were moved from the greenhouse into a rearing room and held at 25 ± 1°C and 55 ± 5% RH. Plants were kept in 47.5- by 47.5- by 93.0-cm BugDorm-4180 F Insect Rearing Cages (Bioquip, Rancho Dominguez, CA) and illuminated with 400-W EYE 4200K BT28 bulbs in Metal Halide Growlights (Hydrofarm, Petaluma, CA) for a photoperiod of 16:8 (L:D) h. Plants were exposed to psyllids for 12–14 d until all five nymphal stages were present.

D. aligarhensis were maintained under the same conditions as described for the rearing room but located in a separate room to prevent contamination of the *D. citri* colony. After the 12–14-d psyllid exposure period, plants were moved to the parasitoid room and placed in a cage with ten newly eclosed female wasps. All wasps were removed after 1 wk. Plants remained in the cage for an additional 2 wk until all parasitoids had eclosed. Emerging parasitoids were collected daily, labeled and held in 50-ml centrifuge tubes (Crystalgen, Long Island NY) with a honey-soaked cotton wick until they were used.

Vital Dyes. Fluorescent and vital dyes have been successfully used as internal markers in various parasitic Hymenoptera and Diptera (Strand et al. 1990, Hagler and Jackson 2001). In an attempt to mark parasitoid eggs and perhaps the emerging larvae to facilitate detection and tracking, possibly without dissection, parasitoids were fed various vital dyes. Acridine orange (AO), blue dextran (BD), Congo red (CR), fluorescein isothiocyanate-dextran (FID), or trypan blue (TB) (Sigma, St. Louis, MO) were added to a 50:50 honey:distilled water mixture at varying concentrations. Acridine orange and fluorescein isothiocyanate-dextran (average molecular weight of 150,000) concentrations were tested at 0.05, 0.1, and 0.5%. Blue dextran, Congo red, and trypan blue were tested at concentrations of 0.1, 2, and 5%. All doses were tested against a control consisting of a 50:50 honey:distilled water mixture. Adult wasps were fed the dyes by applying 7 μl of each solution to a 6-mm-diameter Whatman #1 filter paper disc (Whatman, Bridgestone, England). A treated disc was placed in a 5-ml glass screw top vial with a single newly eclosed adult (<12 h) female wasp. In total, 10 wasps were exposed to each concentration for each of the five dyes. Vials were kept in the dark to prevent photo degradation and were held for 24 h at 25°C and 50% RH. After the 24-h feeding period, the wasps were observed for effects on behavior or survival. Wasps were dissected under an MZ FLIII stereomicroscope (Leica Microsystems, Deerfield, IL), and dyes were detected using a green fluorescent protein (GFP)/DsRed dual filter set (excitation, 470/30 nm and 565/20 nm; barrier, 510 nm and 580; emitter, 535/45 nm and 610/60 nm), a Texas Red filter set (excitation, 560/40 nm; barrier, 610 LP nm; emitter, 630/60 nm), or a GFP Plus filter set (excitation, 480/40 nm; barrier, 510 nm; emitter, 525/50 nm) and photographed.

Larval Development. Newly eclosed adult wasps (<12 h old) were collected and placed directly into one of five identical BugDorm cages with one orange jasmine, *Murraya paniculata* (L.) Jack. plant containing either second-, third-, or 5.5-, 6.5-, and 7.5-d-old fourth-instar nymphs. To control for *D. citri* nymphal development, clean freshly flushing plants were placed into a psyllid rearing cage for 24 h to permit oviposition. After 24 h, all adult psyllids were removed, and each plant was isolated in a separate cage until nymphs reached the desired instar. Between 20 and 30 wasps were placed in each cage for 24 h and then removed. All plants were held under the described rearing room conditions for the duration of the experiment. The developmental stage of psyllid instars was determined using previously published criteria (Liu and Tsai 2000), and instars were confirmed by head capsule width by using a miniscale (Bioquip) based on a published scale (Nava et al. 2007).

Approximately 10–30 nymphs from each instar examined (second, third, or 5.5, 6.5, and 7.5-d-old fourth instars) were dissected every 24 h from the end of the initial exposure to wasps so that at least five parasitoid eggs, larvae, or pupae were collected. Daily dissections were performed through wasp emergence. This process was completed a total of three times, each using a new set of plants and insects. The duration of each developmental stage of *D. aligarhensis* was determined indirectly because of the long ovipositional period required to obtain sufficient numbers of parasitized nymphs and of the necessity to kill the host for the observation. Therefore, the estimated duration of each period was determined by conducting the dissections at 24-h intervals after the removal of the wasps. This meant that the starting population of embryos was 0–24 h old at the time of the first observation. At each dissection, the time when the first members of a specific larval stage appeared and when the last members were present was noted. The median of that period was then taken as the estimated duration for that developmental stage. Dissections were conducted using an MZFLIII stereomicroscope with fluorescent capability and equipped with a DC500 digital camera operated with IM50 software (Leica Microsystems). *D. aligarhensis* eggs, larvae, or pupae were photographed, measured using the microscope's reticule calibrated with a stage micrometer (Unico, Dayton, NJ), and their physical position within the nymph was noted. Length measurements were taken from the anterior to the posterior end of eggs and larvae. Diameter measurements were taken from the anterior of the thorax region of larvae and the widest portion of eggs. When superparasitism was observed, the developmental stage of all larvae was recorded. Superparasitized nymphs were excluded from all developmental analysis.

Freshly dissected larvae were placed in a 2.5% glutaraldehyde solution for up to 1 wk until prepared for scanning electron microscopy. Larvae were double rinsed in double-distilled water; dehydrated by passage from water through 25, 50, and 100% ethanol in succession over 1 h; and stored in 100% ethanol. Specimens were subsequently air-dried, sputter coated with gold-palladium by using a SCD-005 sputter coater (BalTec, Canonsburg, PA), and examined using an S-450 scanning electron microscope (Hitachi, Tokyo, Japan) operating at 10 kV.

To determine whether the reduced developmental time (12 versus 16 d) observed when rearing wasps from 6.5-d-old fourth-instar nymphs affected wasp fertility or life span, the fecundity and longevity of wasps recovered from 6.5-d-old fourth instars were examined using two distinct experiments: 1) to determine longevity, three newly emerged wasps reared from 6.5-d-old fourth-instar nymphs and three reared from 3- to 4-d-old second- to third-instar nymphs were randomly selected and individually isolated in cages held under the rearing room conditions. Each day a fresh orange jasmine clipping infested with second- to third-instar nymphs was provided and the previous days clipping was removed. Cages were checked daily until all wasps died. 2) To determine fertility, three newly emerged wasps reared from 6.5-d-old fourth-instar nymphs and three reared from 3- to 4-d-old second- to third-instar nymphs were randomly selected and individually isolated in cages held under the above-mentioned rearing conditions. Each wasp was provided with an orange jasmine seedling infested with second- to third-instar nymphs. White coffee filters were placed around the base of the stem, resting on the soil surface, to prevent wasps from entering the soil and to aid in locating dead wasps. Every 5 d, the wasp was located and the plant was removed and replaced with a new plant infested with psyllids for oviposition. Used plants were held in a separate cage under the same conditions until wasp emergence. This was repeated until all wasps died. If a wasp was unable to be located, a new one was randomly selected and the procedure repeated. Starting 10 d after first wasp exposure, the number of resulting progeny from each wasp was recorded daily.

Statistical Analysis. Chi-square analysis examining differences in the proportion of nymphal instars superparasitized was conducted using PROC FREQ (SAS Institute 2004). To determine whether there was a significant difference between the length of development for eggs oviposited in 6.5-d-old fourth-instar nymphs, the immature wasp life stages (egg–adult) seen per day were given a numerical value (1–8) and totaled. Numbers assigned were as follows: egg, 1; first-instar larvae, 2; second-instar larvae, 3; third-instar larvae, 4; fourth-instar larvae, 5; prepupa, 6; pupa, 7; and adult wasp, 8. Daily totals were divided by the number of stages seen per day to create a mean. Means per development day per host stage were analyzed using PROC GLM (SAS Institute 2004).

Results

Vital Dye Transfer. Green fluorescence was observed through the abdominal cuticle of all live *D. aligarhensis* wasps fed AO or FID regardless of concentration. Higher concentrations led to brighter fluorescence (data not shown). No color was seen through the abdomen of any live wasp fed CR, BD, or TB regardless of concentration. All wasps fed AO or FID at all concentrations survived with no measurable effect on behavior and they retained some level of fluorescence through 21-d after dye ingestion. No detectable effect on behavior or survivorship resulted from a 24-h period in the presence of honey containing AO or FID at 0.05–0.5% or from 1 and 2% doses of CR, BD, and TB. However, *D. aligarhensis* wasps fed the 5% doses of BD and TB were alive but seemed sluggish and exhibited reduced searching behavior, whereas all wasps fed the 5% dose of CR died within the 24-h feeding period, consistent with previously observed negative effects of dyes (Barbosa and Peters 1970). Nevertheless, upon dissection, no dye regardless of concentration, passed through the insect's gut wall and into the hemolymph. Isolated parasitoid gut tracts were colored, but no other tissues including ova were stained.
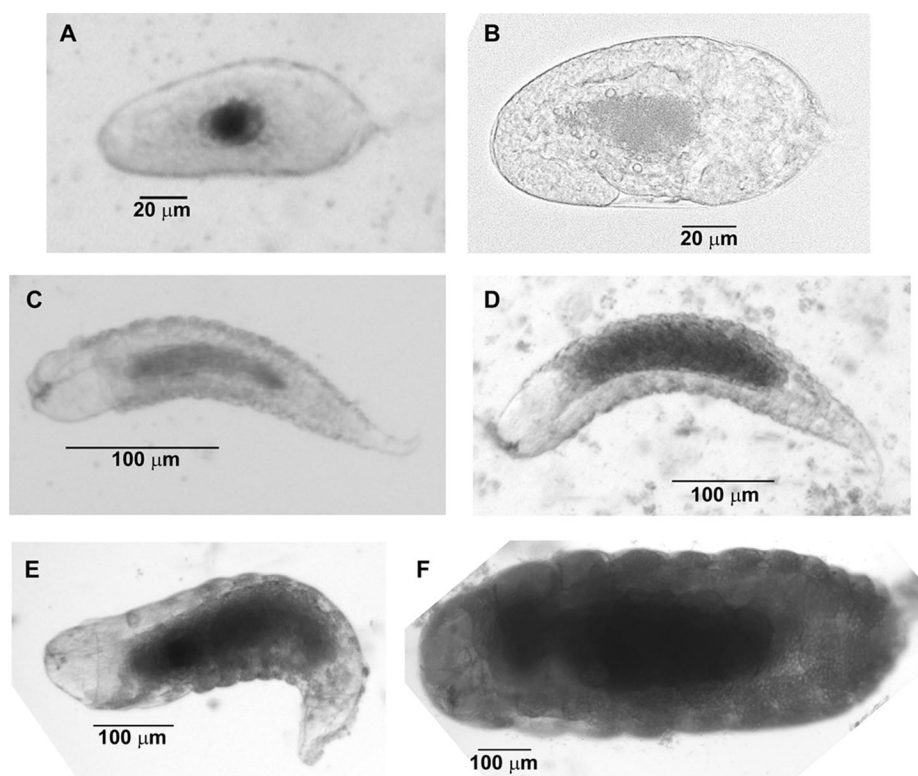
Fig. 1. Egg and larval stages of *D. aligarhensis*. Freshly dissected specimens are all shown oriented with the anterior (head) to the left and posterior (anus) to the right in transmitted light micrographs. The panels show an egg isolated from a host within 12 h after parasitization (A), an egg isolated from a host within 48 h after parasitization (B), a first-instar larva (C), a second-instar larva (D), a third-instar larva (E), and a fourth-instar larva in the prepupal stage (F). Reference bars are shown in each panel to establish magnification.

Larval Development, Morphology, and Axial Orientation. *D. aligarhensis* development included an embryonic stage, four larval instars, a prepupal, and a pupal stage that occurred within the *D. citri* nymphs. Newly laid eggs were a milky white color but cleared within 12 h after oviposition (Fig. 1A). Eggs were oval with an axial length of $130 \pm 10 \mu m$ and a diameter of $100 \pm 1 \mu m$, with the structures of the developing embryo easily discernible within older eggs (Fig. 1B). Embryogenesis required ≈2 d to complete. The oval eggs of *D. aligarhensis* do not share the typical respiratory specialized dumb-bell shape of other encyrtid parasitoids (Quicke 1997). The chorion of the eggs was smooth, thin, and flexible, with no apparent anchoring structures, and the eggs were not found adhering to any host tissues. Eggs were located randomly throughout the host's abdomen and thorax with the majority localized within the abdomen exhibiting no apparent target site or tissue preference. Encapsulation of the eggs was observed in only 1% of the parasitized hosts (five of 500 parasitism events examined). Encapsulated eggs were uniformly dark and completely covered in clear soft tissue (data not shown). One encapsulation event was seen in both second- and third-instar hosts, whereas three were observed in fourth-instar *D. citri*.

All larval stages of *D. aligarhensis* were hymenopteriform and possessed a translucent integument and a weakly developed head capsule (Fig. 1C–F). Larvae taper to a rounded tip at the posterior that became less pronounced as larvae entered their third and fourth instars. All larval stages possessed short, small, reddish orange mandibles that were consistently ≈20% of the total body width (Fig. 2D). No other external appendages, including spiracles or hairs, were observed on first–fourth instars (Fig. 2C). Gut contents were clearly visible through the transparent cuticle (Fig. 1C–F).

First-instar larvae were $410 \pm 7 \mu m$ long with a $100 \pm 5 \mu m$ diameter (Fig. 1C) and had an estimated duration of 2 d. These larvae were unanchored and free moving. Most were found in the foreabdomen or thorax, although several were located in the head region. The orientation of the larval head to the major anterior–posterior axis within the host seemed random.

Second-instar larvae measured $620 \pm 50 \mu m$ long by $180 \pm 2 \mu m$ in diameter (Fig. 1D). The majority of these larval instars were predominantly oriented with their anterior toward the posterior of the host. Larvae were centrally located in the abdomen, thorax, or both.
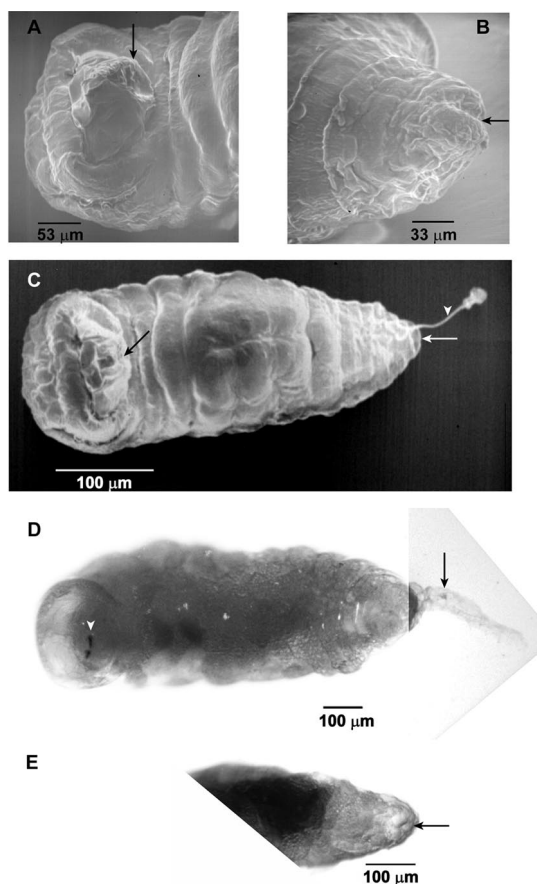
Fig. 2. Detailed structures of the fourth-instar larvae. The mouth and anterior region of a larva were examined in a scanning electron micrograph (A). The black arrow points to the position of the mouth. The anus and posterior region of a larva were observed in a scanning electron micrograph (B). The black arrow points to the position of the anus. The entire ventral surface of the larva was examined in a scanning electron micrograph (C). The black arrow points to the position of the mouth, the white arrow points to the position of the anus, and the white arrowhead points to the attached anal secretions. The ventral surface and internal structures of a larva were observed in a transmitted light micrograph and demonstrate the position of the mandibles (white arrowhead) and the anal secretions (D). The posterior region was cleared of anal secretions and observed in a transmitted light micrograph (E). The black arrow points to the position of the anus. All micrographs show the specimen with the anterior to the left. Reference bars are shown in each panel to establish magnification.

Third-instar larvae measured 860 $\pm$ 70 μm long by 200 $\pm$ 8 μm in diameter (Fig. 1E). The majority of these larvae still maintained their orientation in opposition to the axis of the host with only a few exceptions observed. Larval mouthparts were generally oriented toward the host's ventral surface. Larvae were centrally located within the abdomen or thorax with their posterior often extending into the head region. Most (80%) had the tail anchored to host tissue by anal

secretions which remained attached when the larva was removed (Fig. 2C and D).

Fourth-instar larvae measured 1190 $\pm$ 10 μm long by 400 $\pm$ 20 μm in diameter (Fig. 2D), and all were oriented in opposition to the host with their anterior toward the posterior of the host. The larval mouthparts were generally oriented toward the host's ventral surface. The majority (80%) of those observed were anchored by anal secretions to various host tissues. The last-instar larvae were centrally located and extended through all three host body segments of the *D. citri* nymphs. All late fourth-instar larvae were contained within a fully hardened, darkened mummy of the host by the time they became prepupae. Mummies did not contain any internal host fluids or tissues and were glued to plant surfaces by their ventral surface between the foreabdomen and the head. As the host's exoskeleton began to harden, the wasp larva chewed a small hole in the ventral side of the mummy in the thorax to foreabdomen region. This area remained soft as the rest of the exoskeleton hardened. Liquid drained from this hole and hardened forming a glue-like substance that attached the mummy to the plant surface. Mummies of parasitized nymphs were removed from host plants, and their ventral surfaces were rinsed in distilled water and allowed to air dry. When the internal fluids of unparasitized fourth- and fifth-instar nymphs were painted onto the ventral surface of these mummies and held to various plant tissues, they were unable to stay attached regardless of the drying time. This suggests that host fluid from unparasitized nymphs is not capable of fastening mummies to plant tissue and an additional component from the *D. aligarhensis* larva is essential to produce the "glue" that attaches the parasitized mummy to the plant surface.

Larvae entering prepupation shorten in length by constriction to 1,070 $\pm$ 40 μm long by 510 $\pm$ 12 μm in diameter, forming uniform segments often using only 70–80% of the length of the psyllid mummy (Fig. 1F). All pupae were exarate and positioned in the opposite direction to the host axis, with the head turned downward facing the host's ventral surface. The structural definition of the head and abdomen were observed within 2 d after pupation with the legs, mouthparts, and antennae becoming distinguishable by day 4 of the pupal stage (Fig. 3A). Subsequently, the thorax, eyes, and ocelli had begun melanization by 5 d after pupation that was complete in the abdomen, legs, and lastly the antennae by 7 d after pupation. In addition to increased pigmentation, wing venation, and setae development occurred at this time as well (data not shown).

Three events of adult eclosion were observed during this study. The pharate adult began eclosion with the ventral surface oriented toward the ventral surface of the nymphal mummy. Rotational movements reoriented eclosing adults 180° so that the ventral surface finally resided facing upward toward the host's dorsal surface. The eclosing adult then chewed through the dorsal abdominal integument of the *D. citri* mummies, leaving a round emergence hole (Fig.
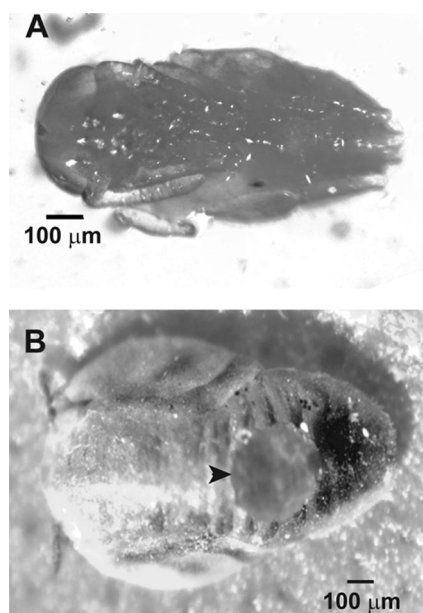
Fig. 3. Ventral view of a pharate adult wasp (A) with the head (anterior) oriented to the left. The mummified remains of a *D. citri* nymph (B) was observed to show the exit hole in the dorsal abdominal cuticle used by the eclosing *D. aligarhensis* adult (the mummy is oriented with the anterior to the left; the black arrowhead points to the anterior edge of the exit hole). Reference bars are shown in each panel to establish magnification.

3B). Once the emergence hole was carved, the eclosing adults were able to crawl out onto the plant surface where they groomed and flexed their wings, rested, and then began walking.

Concomitant Host and Parasitoid Development. Liu and Tsai (2000) determined that *D. citri* nymphs reared at 25°C enter the fourth instar, on average, 5.5 d after egg hatch and enter the fifth instar ≈4.5 d later on day 9. This is in comparison with a duration of 1.5 d for both the second- and third-instar nymphs. Although the fifth instar has the longest duration of ≈5 d, the fifth-instar *D. citri* nymphs are not used by *D. aligarhensis* for parasitism or feeding purposes.

After a parasitism event by *D. aligarhensis*, *D. citri* nymphs continued to feed, develop, and molt through to the fifth instar regardless of which instar (second–fourth) was parasitized. Parasitized nymphs remained as fifth instars until they began to harden to form a mummy.

For *D. aligarhensis* that were oviposited in second- or third-instar *D. citri* nymphs, the embryonic, first, and second larval instars lasted an estimated 2 d each (Fig. 4A). The duration of the third- and fourth-instar larvae and prepupal stages was 1 d each followed by a 7-d pupal stage. Nymphal mummies formed at 8 d after parasitism coinciding with the beginning of the prepupal stage for the *D. aligarhensis* larvae, and adult eclosion occurred 16 d after oviposition.

In contrast, when oviposition occurred in fourth-instar hosts, developmental time for *D. aligarhensis*
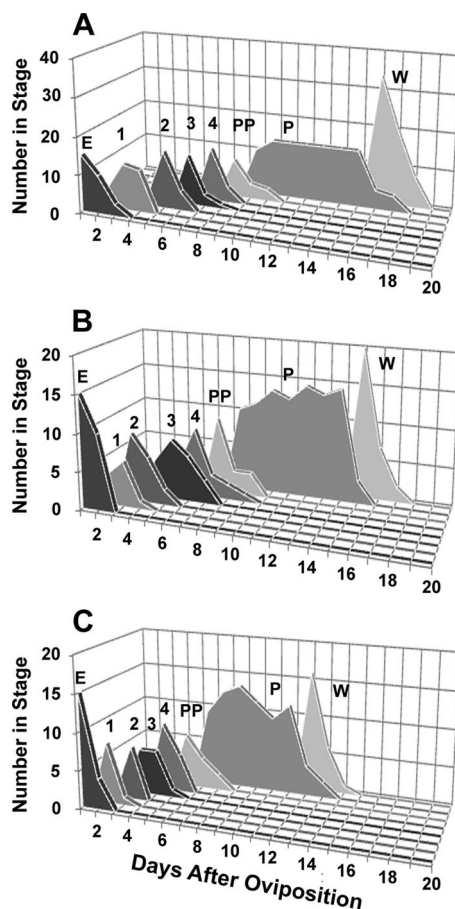

Fig. 4. Estimated temporal pattern for the developmental stages of the wasp *D. aligarhensis* oviposited in various stages of host nymphs. The developmental periods for wasp progeny whose eggs were oviposited in third-instar *D. citri* nymphs are shown in A. The profile for eggs deposited in second-instar nymphs was not included because there was no discernible difference between the data for second- and third-instar nymphal oviposition. The developmental periods for wasp progeny whose eggs were oviposited in early (day 5.5) fourth-instar *D. citri* nymphs are shown in B. The developmental periods for wasp progeny whose eggs were oviposited in mid (day 6.5)-fourth-instar *D. citri* nymphs are shown in C. The designation above the curve indicates the stages of development. E, embryo; 1, first-instar larvae; 2, second-instar larvae; 3, third-instar larvae; 4, fourth-instar larvae; PP, prepupae; P, pupae; W, wasps.

from egg to adult eclosion was radically shortened and the time depended on the host age. When 5.5-d-old fourth-instar *D. citri* nymphs were parasitized, developmental time from the egg through the fourth instar was slightly shortened (1 d) compared with those developing from parasitized second- or third-instar nymphs with no discernible difference in the duration of the prepupal or pupal stages (Fig. 4B). Mummies were seen 7–8 d postparasitism with adult eclosion on day 15, 1 d less than for second or third instars (Fig. 4B). However, when parasitization occurred in 6.5-d-old fourth-instar *D. citri* nymphs, mummification was

observed within 5–6 d and adult eclosion occurred on day 12 (Fig. 4C). Development (egg–pupa) of immature *D. aligarhensis* was clearly shortened when oviposition occurred in these older nymphs. A significant difference in the overall developmental time between host stages was confirmed for eggs oviposited in 6.5-d-old fourth-instar *D. citri* nymphs compared with the developmental times observed for eggs oviposited in the earlier *D. citri* nymphs ($F = 8.74$, df $= 1$, $P = 0.0044$). If oviposition occurred in 7.5-d-old fourth-instar nymphs, no wasps were recovered. Eggs were found up to 6 d after parasitism in these *D. citri* nymphs but seemed darkened, with little or no embryonic development.

No significant difference was seen in either life span ($F = 0.04$, df $= 1$, $P = 0.85$) or fertility ($F = 0.08$, df $= 1$, $P = 0.79$) between the short development (12 d) and normal wasps (16 d). Wasps reared from 6.5-d-old fourth-instar nymphs lived for an average of 26.3 ± 2.9 d (SE) and produced an average of 185.3 ± 31.7 offspring. Those reared from 3- to 4-d-old second—third-instar nymphs lived for an average of 25.6 ± 1.48 d and produced 175 ± 21.5 offspring.

Superparasitism. Of the >500 wasp eggs, larvae or pupae located in *D. citri* nymphs, only 22 events of superparasitism (4%) were observed. Ten were observed in second-instar nymphs, four in third-instar nymphs, and eight in fourth-instar nymphs (four in 5.5-d-old nymphs and four in 6.5-d-old nymphs). Chi-square analysis showed no significant difference in the proportion of second–fourth instars that were superparasitized ($\chi^2 = 2.5$, df $= 2$, $P = 0.28$).

Of the 10 events in second instar hosts, six consisted of two eggs in one host (all within the abdomen). Four events were instances of two larvae in one host where one larva was a first instar, whereas the other was either a second-, third-, or fourth-instar larva. All more developmentally advanced larvae seemed healthy, whereas the first-instar supernumeraries were alive but seemed sluggish or motionless.

Superparasitism of third-instar nymphs resulted in events of two eggs in two different hosts, and one instance each of one egg plus a first-instar larva and one first-instar larva plus a third-instar larva. Two 5.5-d-old and two 6.5-d-old fourth-instar hosts contained two eggs each, and another two hosts each contained a first-instar larva plus a third-instar larva. No superparasitism events were observed in 7.5-d-old fourth-instar hosts.

Discussion

The immature stages of *D. aligarhensis* progressed through a larval–pupal development that is typical for many endoparasitoids (Quicke 1997). Initially, the eggs and larvae were free floating within the hemocoel, but by the third instar the larvae had begun attaching to the nymphal tissues via anal secretions. This attachment provided a means of orienting within the host nymph and the last-instar wasp larvae were found with their posterior anchored in the thoracic region and their head oriented toward the posterior of

the host nymph. As the *D. aligarhensis* larva enters the prepupal stage, parasitized nymphs begin to harden into a mummy. The wasp larva must attach the mummy to plant tissue. This is accomplished by chewing a hole in the host's ventral surface where excess host body fluid, and probably the larva's secretions, drain and harden forming a glue-like substance that secures the mummy to the plant surface. In contrast, the prepupa of *T. radiata* spins a silk webbing to secure the mummy to the plant surface (Patil et al. 1993; P.A.S., unpublished data).

Once pharate adult development has completed, the eclosing adult *D. aligarhensis* wasp must exit the mummy by chewing a round hole through the dorsal surface of the host's abdominal cuticle similar to the behaviors of other endoparasites (Liu and Stansly 1996). This exit hole can be used to distinguish between a mummy parasitized by *D. aligarhensis* from *T. radiata* that exits dorsally through the thorax (Hoy 2005) rather than the abdomen.

The observation that *D. aligarhensis* larvae were anchored to tissues of the *D. citri* nymphs by secretions originating from the anus of the larvae is the first report of this manipulation of the host environment and represents a unique modification within the hymenopteran family Encyrtidae. Anal secretions have been observed in other parasitoids but have been associated with other functions. The larva of the endoparasitic wasp *Pimpla turionellae* L. (Hymenoptera: Ichneumonidae) produces a droplet of anal secretion that directly inhibits the DOPA-tyrosinase reaction, thus blocking melanization and encapsulation as well as preventing the growth of bacteria and fungi (Fuhrer and Willers 1986). A clear, sticky anal secretion was observed as a means of fastening the freshly ecdysed pupa of *Elachertus scutellatus* Howard (Hymentoptera: Eulophidae), an ectoparasitoid of *Calpodes ethlius* Stoll (Lepidoptera: Hesperiidae), directly to its leafy substrate (MacDonald and Caveney 2004). In addition, many chalcidoids, including some endoparasitoids, secrete a protective silk cocoon-like structure from the anus of the final instar (Quicke 1997). In contrast to the anal secretions, the anal vesicles protruding from early instars of some braconids and ichneumonids are responsible for various interactions with their hosts, such as secretory and uptake functions (respiration, nutrition, water and ion balance) and excretion (Edson and Vinson 1976, 1977; Kaeslin et al. 2006; Yu et al. 2008).

The total time that this population of *D. aliagrhensis* required to completed development was 16 d at 25°C when the eggs were oviposited in second and third-instar *D. citri* nymphs. In comparison, the previously imported Taiwan population of *D. aligarhensis* required 18 d at 25°C (Skelley and Hoy 2004). However, when oviposition occurred in 6.5-d (mid)-old fourth-instar *D. citri* nymphs, adult eclosion of *D. aliagrhensis* occurred only 12 d after oviposition, which shortened development by 25%. Importantly, the reduced developmental period did not have an effect on either the fertility or life span of the wasp. This finding suggests that commercial production of *D. aligarhensis*

for augmentative release could be enhanced by providing exclusive oviposition with mid fourth-instar *D. citri* nymphs.

Wasps were unable to effectively parasitize 7.5-d-old fourth instar hosts. At this age, nymphs are nearly fifth instars, so this finding is not surprising. As nymphs age, particularly during the fourth instar, their cuticles thicken and they become more efficient at defensive twitching. These changes make it more difficult for wasps to successfully oviposit and are probably are responsible for the low number of eggs located in the 7.5-d fourth-instar nymphs ($n = 10$). In addition, all eggs found oviposited in late fourth-instar *D. citri* nymphs were dead, which suggested there may be a physiological restriction to wasp development in older nymphs. It was not determined whether older fourth-instar hosts are capable of suppressing egg hatch, or if some chemical or hormone in host hemolymph needed for development is lacking.

This population of *D. aligarhensis* exhibited low levels of superparasitism (<5%) despite large numbers of wasps (20–30 per plant per instar) being released into the same cage. However, the exposure period was limited (24 h), so superparasitism rates would probably increase with increased exposure times. No more than two larvae were observed in any one host nymph. In all superparasitism events, one larva was more developmentally advanced and the other larva seemed to have remained as a first instar. Supernumerary conspecific larvae occurring in superparasitism are subject to competitive elimination either by physical attack or by physiological suppression of development (Fischer 1971). Typically, first-instar larvae of other endoparasitic species are equipped with large mandibles capable of destroying conspecifics (Salt 1961, Quicke 1997). However, the mandibles of *D. aligarhensis* are small and short, and no physical damage was observed in any coexisting supernumerary larvae, suggesting a physiological suppression of limiting supernumerary development. Several forms of physiological suppression have been proposed, including the larval release of toxins or cytolytic enzymes, anoxia, nutritional deprivation by older instars, changes in nutrient availability, or by substances injected by the adult parasitoid during oviposition (Fischer 1971, Vinson and Iwantsch 1980). Unfortunately, there was no transference of orally administered vital or fluorescent dyes to the eggs, so noninvasive observation of superparasitism was not possible. Because dissection was the only means of identifying superparasitism events, it was impossible to determine whether the larger of the two larvae would successfully complete development to adulthood.

The observations on development of *D. aligarhensis* provide a foundation for developing informed strategies for rearing of this endoparasite for augmentative release to control the Asian citrus psyllid. That the temporal period for completing development can be shortened by 25% should facilitate a more robust rearing program for the production of *D. aligarhensis* wasps.

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