

***Doryctobracon areolatus* (Hymenoptera, Braconidae) a parasitoid of early developmental stages of *Anastrepha obliqua* (Diptera, Tephritidae)**

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Abstract

Natural parasitism of *Doryctobracon areolatus* (Szépligeti) (Hymenoptera: Braconidae) on various developmental stages of *Anastrepha obliqua* (Macquart) (Diptera: Tephritidae) attacking *Spondias mombin* L. fruits was studied under field conditions. We collected 120 fruits of *S. mombin* from which we got 495 *A. obliqua* larvae of different instars. A total of 88% of these larvae were parasitized. Within the parasitized cohort, the first-instar of *D. areolatus* was frequently detected in all 3 larval stages (L1 = 94.3%, L2 = 98.1%, and L3 = 100%), and the rest (i.e., L1 = 5.7%, L2 = 1.8%) corresponded to the presence of eggs. In fruits with controlled infestation and cage-induced parasitism under field conditions, *D. areolatus* oviposited in mature eggs and recently hatched larvae of *A. obliqua* with comparable frequencies. Seven preimaginal stages of *D. areolatus* were observed during their development, which was completed in 27 days. It is concluded that *D. areolatus* has the capacity to oviposit in embryo eggs and neonate larvae of *A. obliqua* and that its first-instar larvae (with three distinct sizes) are capable of synchronizing their development with the development of the host larvae. This finding represents the first report of a native parasitoid attacking eggs or neonate larvae of a tephritid in the Neotropics. The implications of this finding are discussed within the context of the competitive interactions of this species with other parasitoid species under sympatric conditions, as well as the relevance for developing laboratory rearing methods for biological control purposes.

Keywords

Egg parasitoid, laboratory breeding, interspecific competition, morphology, fruit flies, biological control

Introduction

The native parasitoid guild that attacks fruit flies of the genus *Anastrepha* Schiner in the Neotropics is mainly composed of a group of solitary koinobiont endoparasitoids (primarily Braconidae and Figitidae) that oviposit in the host larvae and emerge from the pupae. The genus *Doryctobracon* constitutes 27% of the parasitoid species and shares a closely related evolutionary history with *Anastrepha* (López et al. 1999, Ovruski et al. 2000).

Another smaller group of *Anastrepha* parasitoids are pupal idiobionts that attack their hosts when they are in the soil and is represented by five species within the genera *Coptera* and *Trichopria* (Diapriidae) and three polyphagous species, *Pachycrepoideus vindemiae* (Rondani), *Spalangia cameroni* Perkins, and *S. endius* Walker (Pteromalidae) (Ovruski et al. 2000). The parasitoid guild of *Anastrepha* spp. in the Neotropics represents an important source of species with potential to be used in biological control programs against native tephritids (Aluja et al. 2003). However, native *Anastrepha* parasitoids attacking eggs or early larval developmental stages have not been reported. The only report of a parasitoid attacking *Anastrepha* eggs in the Americas correspond to Wharton et al. (1981), who found a small level of parasitism of the introduced *Fopius arisanus* (Sonan), apparently on *A. striata* (Schiner) eggs.

According to Aluja et al. (2009) species of the genera *Doryctobracon*, such as *D. crawfordi* (Viereck) and *D. areolatus* (Szépligeti), exhibit great potential as biological control agents and should be examined from the point of view of mass rearing projects. *D. areolatus* has been reported as a solitary koinobiont endoparasitoid that attacks third instar larvae of *Anastrepha* spp., both in native and exotic commercial fruits, with a wide distribution from Florida to Argentina (Hernández-Ortiz et al. 1994, López et al. 1999, Ovruski et al. 2000, Sivinski, et al. 2000, Aluja et al. 2003, 2009). This species frequently shows field dominance among concurrent parasitoid species attacking *Anastrepha* spp. (López et al. 1999, Sivinski et al. 1997, 2000; Aluja et al. 2003, Ovruski et al. 2004). The presence of diapause (Aluja et al. 1998, Ovruski et al. 2004) and an extrinsic capacity to find patches with low host density (Sivinski et al. 1998) allows an ample distribution in regions with low plant diversity (Eitam et al. 2004).

In the central region of Veracruz, Mexico, *D. areolatus* is the most abundant parasitoid species attacking *Anastrepha obliqua* (Macquart) in *Spondias* spp. (Cabrera et al. 2006), where it has frequently been observed at the beginning of the fruiting season. This suggests that this species could parasitize earlier developmental stages in relation to sympatric parasitoid species. Normally, parasitoids attacking eggs and first instar larvae of their hosts become more competitive than those attacking later stages (Wang and Messing 2002, Wang et al. 2003, Wang et al. 2008, Argov et al. 2011), showing

important potential to be used as biocontrol agents. Therefore, our aims in this study were: 1) to determine if *D. areolatus* parasitize immature stages of *A. obliqua* and 2) to characterize its developmental stages during each phase of host development.

Materials and methods

Study area

The study was conducted in the coastal region of central Veracruz, which is characterized by high densities of *A. obliqua* hosts, such as mango (*Mangifera indica* L.), native *Spondias* species and guavas (*Psidium guajava* L.). Fruit samples were collected from trees located in backyard orchards and marginal zones, which provide resources for the presence of flies and parasitoids all year round. This zone is located between 19°00' and 18°55' North latitudes and 96°10' and 96°13' West longitudes, with a mean altitude of 18.5 m.a.s.l. The climate is semi-humid, with a mean annual rainfall of 1,358 mm and a very marked rainy season from June to September. The highest mean monthly temperature (29.1 °C) occurs in the month of June and the lowest mean monthly temperature (21.4 °C) occurs in the month of January (SMN 2010).

Determination of the natural parasitism of *A. obliqua* larvae by *D. areolatus* under field conditions

From September to October 2013, hog plum (*Spondias mombin* L.) fruits were collected from four sites, three in the locality of “El Copital” and one in “El Mangal”, municipality of Medellín de Bravo, from four to five trees per site. Fruits were collected directly from the trees (36 fruits, 30%) and from the ground surrounding the trees (84 fruits, 70%). Each sample consisted of 10 fruits per site. Samples were in three sampling dates separated by seven days to cover the fruiting season of *Spondias* spp. A total of 120 fruits were dissected.

Anastrepha obliqua larvae were extracted from each of the fruits the same day they were collected. Larval instars were categorized based on the width of the cephalic capsule and the body length (mean \pm SE) (Carroll and Wharton 1989). Larvae were dissected immediately after collection, and the frequencies of the immature stages of *D. areolatus*, or any other parasitoid species, were recorded following descriptions by Aluja et al. (2013) and Murillo et al. unpublished data.

Photographs were captured with a Motic Plus 2.0° camera connected to a Carl Zeiss Smz -168° stereomicroscope. The *D. areolatus* immatures inside the *A. obliqua* larvae were measured using Motic Imagen Plus 2.0 ° software. The percentage of parasitized larvae was calculated, and frequencies of immature *D. areolatus* stages per larval instar of *A. obliqua* were determined.

Induction of *D. areolatus* parasitism on *A. obliqua* eggs and recently hatched larvae

Wild *A. obliqua* flies were collected as larvae from infested *S. mombin* fruits in the field. Upon completion of their development, the larvae were placed in containers with sterile sand for pupation. They were maintained under these conditions until adult emergence. Adults were maintained with water and food (sugar plus hydrolyzed yeast in a 3:1 ratio) until they were sexually mature.

Hog plum (*S. mombin*) fruits were previously protected from natural infestation by bagging clusters of young fruits using 30 × 20 cm brown paper bags. A total of 30 bags (≈10 fruits/bag) were used to protect ≈300 fruits. The fruits were subsequently collected, taking their maturity into account to allow for experimental infestation.

Infestation on the previously protected fruits was induced by exposing the fruits to *A. obliqua* gravid females (8–10 days old) in Plexiglass cages (20×20×20 cm) placed on a table in the field. Two clusters with five to eight fruits were placed in each cage together with 10 female flies and remained in the field at a mean temperature of 28.2 °C (range: 23.2–36.1) and a mean RH of 81.6% (range: 55.1–95.3). Flies were maintained for six hours in each cage and dead flies were replaced.

Anastrepha obliqua eggs were exposed to the parasitoid in the same type of cages 24, 48, and 72 hours after fly oviposition in the fruit (≈egg age), in order to cover the different egg stages before larval hatching. Twenty 7-day-old *D. areolatus* females were placed in each cage for three hours, time enough to locate and oviposit in the exposed eggs. Immediately after exposure, 35 *A. obliqua* eggs and 15 newly hatched larvae were extracted from the fruits. The eggs were characterized as either yolk-egg or embryo-egg (after Chapman 2013, pp: 358–407). All of the *A. obliqua* individuals in the egg and larval stages were dissected to characterize and record the immature stages of *D. areolatus*.

Characterization of *D. areolatus* development and morphological changes

Development of *D. areolatus* eggs and larvae was individually photographed and measured. To follow the development of *D. areolatus* in *A. obliqua* pupae, mature *A. obliqua* larvae were obtained from presumably infested fruit collected in the field. These larvae were placed in 100-ml plastic containers with sterile sand as a substrate to facilitate pupation. Three cohorts of 50 *A. obliqua* pupae were examined, and 3 to 5 pupae per day were dissected from 0 to 12 days of growth. *D. areolatus* individuals and their developmental stages were recorded for each *A. obliqua* pupa.

The frequencies of the different immature developmental stages and the characteristics of *D. areolatus* were calculated for each immature stage of *A. obliqua*. All of the observations of the organisms were conducted using the above-mentioned microscope.

Data analysis

Chi-square test was used to compare the number of *D. areolatus* individuals observed at each developmental stage with the expected number, using SPSS Statistic 17.0. (SPSS Inc., 2008). Measurements of the cephalic capsules of *A. obliqua* larvae are given as the mean \pm (SE). The proportions of the immature stages of *D. areolatus* for each *A. obliqua* egg and larval stages are presented as observed numbers, and *D. areolatus* development is presented as numbers of individuals and percentages.

Results

Natural parasitism in the field

From the 120 fruits that were sampled, 495 *A. obliqua* larvae were extracted; 85, 115 and 295 of these were L₁, L₂ and L₃ larvae, respectively, and 69 (82%), 104 (90%) and 264 (89%) of these larval stages were parasitized, respectively (mean parasitism = $88 \pm 5.2\%$).

D. areolatus was the dominant parasitoid species (93.1%), and only *Utetes anastrephae* (Viereck) (5.4%) was found as the second most dominant parasitoid (Figure 1a). The remaining 1.5% (third-instar larvae) was parasitized by both species and no apparent advantage was observed for either species, except for the occasional larger size of *D. areolatus* larvae (Figure 1b). *D. areolatus* and *U. anastrephae* larvae found together were first-instar larvae, which were easily distinguishable from each other, primarily because of the larger sizes of the cephalic capsule and the jaws of *U. anastrephae* (see Fig. 1).

The mean \pm (SE) of the widths of the cephalic capsules and the body lengths, respectively, of *A. obliqua* larval instars were 0.09 ± 0.001 mm and 0.90 ± 0.05 mm for the L₁, 0.37 ± 0.03 mm and 4.67 ± 0.3 mm for the L₂, and 0.63 ± 0.004 mm and 9.16 ± 0.3 mm for the L₃.

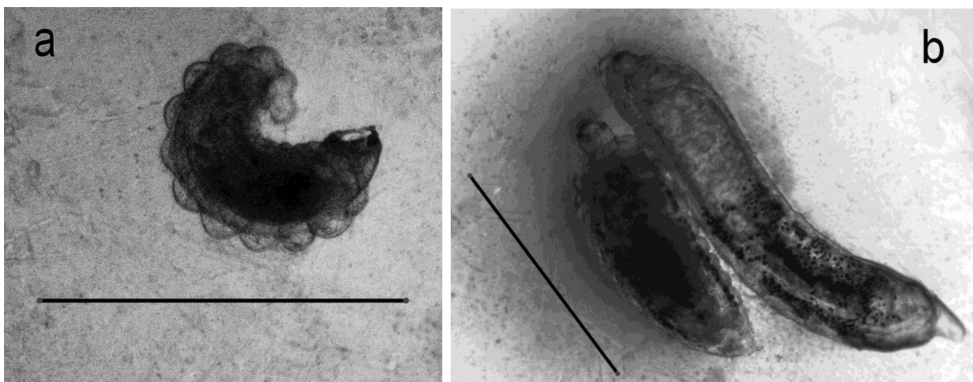


Figure 1. Parasitoids found in naturally parasitized *A. obliqua* larvae. **a** First instar larva of *U. anastrephae* found in a third instar larva of *A. obliqua* and **b** Larvae of *U. anastrephae* and *D. areolatus* found together in a third instar larva of *A. obliqua*. Scale bars = 1 mm.

Table 1. Numbers of observed individual stages of development of *D. areolatus* recorded in the different larval stages of *A. obliqua* extracted from field-collected hog plums (*S. mombin*).

<i>D. areolatus</i>	<i>A. obliqua</i>		
	L1	L2	L3
Egg	3	2	0
L ₁ Early	66	5	0
L ₁ Intermediate	0	97	2
L ₁ Late	0	0	262

L1 Early = First instar larva small (≈ 0.8 mm long); L1 Intermediate = First instar larva medium (≈ 1.4 mm long); L1 Late = First instar larva large (≈ 1.7 mm long).

The numbers of the developmental stages of *D. areolatus* recorded in the various stages of naturally parasitized *A. obliqua* are given in Table 1. Embryo-eggs and a high frequency of early first-instar larvae were detected in *A. obliqua* first-instar larvae. In second-instar larvae, the presence of *D. areolatus* eggs was minimal, with a higher frequency of intermediate first-instar larvae. In the third-instar larvae, nearly all of the *D. areolatus* were late first-instar larvae. The relationship between the immature stages of *D. areolatus* and the larval states of *A. obliqua* was significant ($\chi^2_4 = 800.9, P < 0.0001$).

Presence of *D. areolatus* eggs in recently hatched *A. obliqua* larvae

In the controlled infestation experiment, *A. obliqua* yolk-eggs were not parasitized, which indicates that *D. areolatus* did not parasitize eggs without a formed embryo (Table 2). However, parasitism was detected in *A. obliqua* embryo-eggs. A recently laid *D. areolatus* egg (with yolk in its interior) on an *A. obliqua* embryo can be observed in Figure 2 and is folded in the embryo’s interior, given that both structures are of a similar length. Seven recently hatched *A. obliqua* larvae that were parasitized by *D. areolatus* eggs were dissected; five were still in the yolk stage and two in the embryo stage (Figure 3a).

Table 2. Numbers of *D. areolatus* developmental stages found in *A. obliqua* eggs and first instar larvae when *S. mombin* fruits were infested in a controlled manner.

<i>D. areolatus</i>	<i>A. obliqua</i>			
	Eggs		First instar larvae	
	Yolk	Embryo	Newly emerged	Mature
Egg (yolk)	0	3	5	0
Egg (embryo)	0	0	2	2
L ₁ Early	0	0	0	13

L1 Early = First instar larva small (≈ 0.8 mm long).

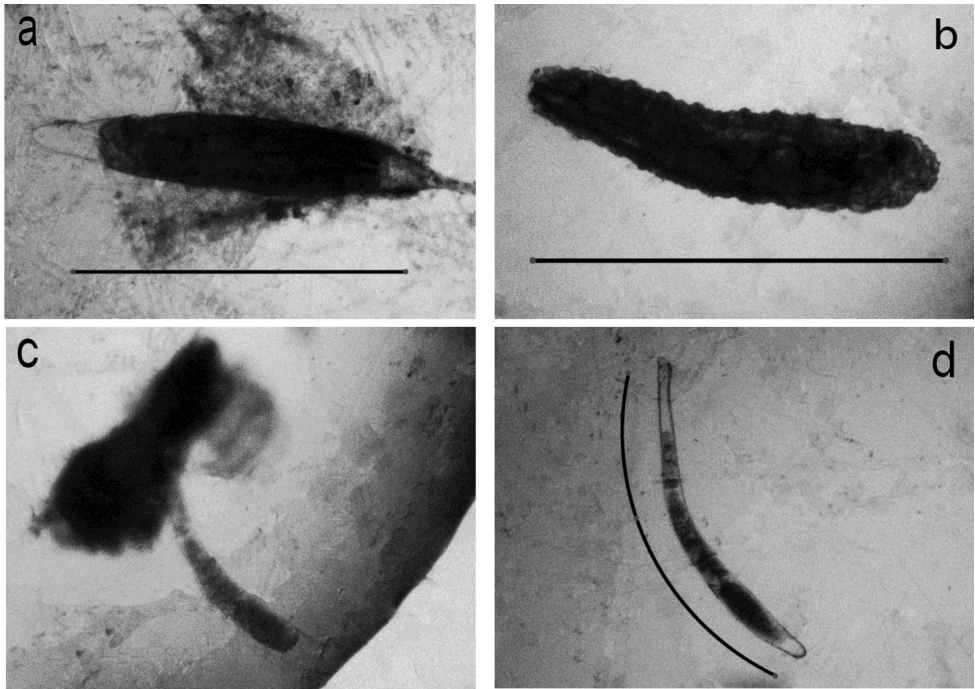


Figure 2. *D. areolatus* parasitizing an *A. obliqua* embryo. **a** *A. obliqua* egg embryo **b** *A. obliqua* embryonic egg removed **c** *D. areolatus* egg extracted from *A. obliqua* embryo, and **d** *D. areolatus* egg. Scale bars = 1 mm.

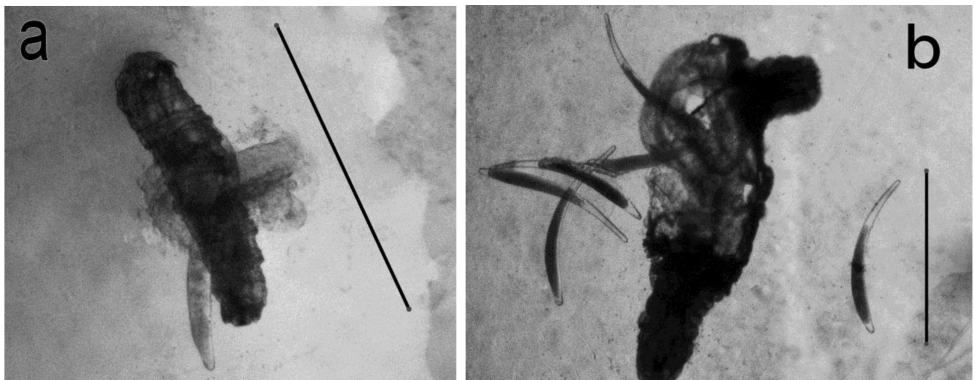


Figure 3. Parasitized *A. obliqua* newly hatched larvae **a** with a *D. areolatus* egg inside and **b** with six eggs of *D. areolatus* inside. Scale bars = 1 mm.

Superparasitism by *D. areolatus* was recorded in two out of seven recently hatched *A. obliqua* larvae, and six parasitoid eggs were recorded from one larva (Figure 3b).

Fifteen mature first-instar *A. obliqua* larvae were dissected. Of these, 13 were parasitized with early first-instar larvae and 2 with embryo-eggs of *D. areolatus*. Yolk-eggs of *D. areolatus* were not found (Table 2). The relationship between the immature stages of *D. areolatus* and the immature stages of *A. obliqua* was significant ($\chi^2_4 = 22.4$, $P < 0.0001$).

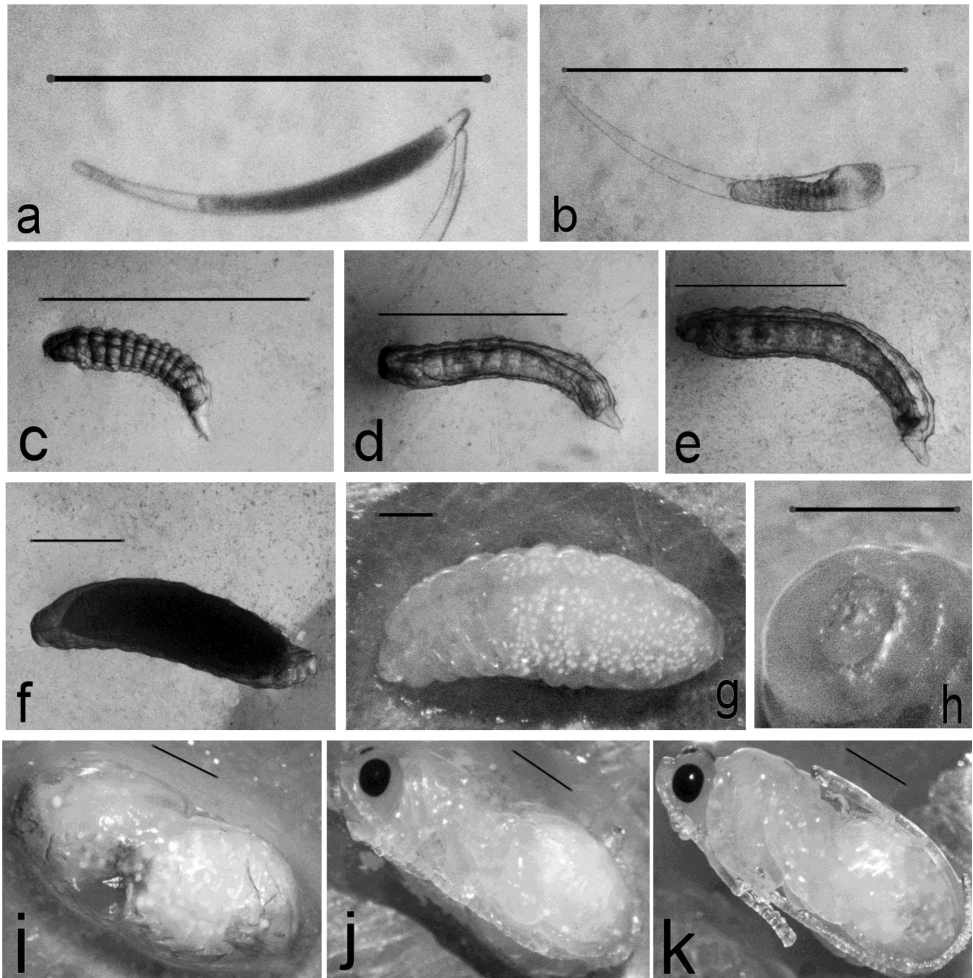


Figure 4. Development of immature stages of *D. areolatus*. **a** egg yolk **b** egg embryo **c** early first instar larva **d** intermediate first instar larva **e** late first instar larva **f** second instar larva **g** third instar larva **h** jaw of third instar larva **i** prepupa **j** male pupa, and **k** female pupa with her ovipositor. Scale bars = 1 mm.

Characterization of *D. areolatus* development

The *D. areolatus* egg measures ≈ 1 mm long and has an elongated shape and a whitish color with a dark yolk. After 24 hours, the embryo is formed with a claviform appearance and measures ≈ 0.5 mm in length (Table 3 and Figure 4).

The embryo of *D. areolatus* becomes an early first-instar larva within 24 to 36 hours of oviposition and measures ≈ 0.8 mm in length. After three to four days, an early first-instar larva grows into an intermediate first-instar larva, with a length of ≈ 1.4 mm. After another three to four days, the larva grows into a late first-instar larva measuring ≈ 1.7 mm in length. This larva almost immediately changes to a second-instar once the host pupa has formed, increasing in size and changing its shape (Table 3 and Figure 4).

Table 3. Numbers (and percentages) of individuals of each developmental stage of *D. areolatus* during the various developmental stages of *A. obliqua* in *S. mombin*.

<i>D. areolatus</i>	<i>A. obliqua</i>													n (state Biological)								
	Egg			Larva			Pupa															
	Yolk	Embryo		L ₁	L ₂	L ₃	0	1	2	3	4	5	6		7	8	9	10	11	12	13	
Egg	0 (0)	3 (60)		2 (40)																		5
Embryo				5 (100)																		5
L ₁ Early				66 (93)	5 (7)																	71
L ₁ Intermediate					97 (98)	2 (2)																99
L ₁ Late						262 (84)	5 (16)															267
L ₂							1 (5)	10 (45)	6 (27)	5 (23)												22
L ₃											8 (57)	6 (43)										14
Prepupa													10 (48)	8 (38)	3 (14)							21
Pupa															17 (47)	8 (22)	7 (19)	4 (11)				36
Pharate adult																			5 (23)	17 (77)		22
Total 562																						

L₁ Early = First instar larva small (≈ 0.8 mm long), L₁ Intermediate = First instar larva medium (≈ 1.4 mm long) and L₁ Late = First instar larva large (≈ 1.7 mm long).

In recently formed *A. obliqua* pupae, a higher frequency of late first-instar larvae of *D. areolatus* in the process of transformation to the second instar stage were observed. In 1-day old pupae, the *D. areolatus* larva had changed completely to the second instar stage, measuring ≈ 3.5 mm long, losing the cephalic capsule and occupying more than a third of the host pupa. In 3-to-4-day old pupae, the larva develops into the third instar stage, measuring ≈ 6.0 mm long, changing body shape, and occupying all of the host pupa. In 6 day-old *A. obliqua* pupae, *D. areolatus* pre-pupae that exhibit eye development have formed. In 8-day old host pupae, the parasitoid pupae are already observed with well-defined structures and genitalia. In 12-day old and older *A. obliqua* pupae, the parasitoids are found as their complete adult structure (Table 3 and Figure 4).

Discussion

The presence of eggs and larvae of *D. areolatus* in the interior of eggs and recently hatched *A. obliqua* larvae represent a novel finding within the native parasitoid guild that attack fruit flies in the Neotropics, because there have been no previous reports of any native parasitoid covering this ecological niche (López et al. 1999, Ovruski et al. 2000).

Among the particular observations regarding this finding under forced conditions, it was notable that *D. areolatus* oviposits inside embryo-eggs of *A. obliqua*, depositing a flexible egg that can fold inside the interior of the host embryo, and that the first-instar larvae present a prolonged development with three distinct sizes that synchronize with the development of the host larva and pupa. The low number of embryo-eggs found with egg parasitoids could be explained by the short developmental time of eggs (less than 24 hours).

In Mexico, *D. areolatus* has been reported to be closely associated with *A. obliqua* in fruit hosts of the genus *Spondias* (López et al. 1999, Ovruski et al. 2000, Sivinski et al. 2000), which most likely is favored by the presence of semiochemicals that could allow it to attack early stages of *A. obliqua* (eggs and recently hatched larvae). This scenario seems similar to that of *F. arisanus*, which detects marking pheromones and kairomones that emanate from the eggs of its host or from the interaction of the fruit and the host egg (Rousse et al. 2005, 2007, Pérez et al. 2013).

The finding of the parasitism of *D. areolatus* on eggs and recently hatched larvae of *A. obliqua* sheds light on two relevant aspects of its role as a natural enemy and biological control agent of fruit flies: 1) its competition and coexistence with other opiine parasitoids, highlighting the exotic species *Diachasmimorpha longicaudata* (Ashmead) and the native species *U. anastrephae* (García-Medel et al. 2007, Sivinski et al. 1998, Paranhos et al. 2013, Aluja et al. 2013), and 2) the promising development of its mass rearing under laboratory conditions (Eitam et al. 2003, Aluja et al. 2009).

It has been argued that *D. areolatus* is an inferior competitor compared to *D. longicaudata* (Sivinski et al. 1998, Eitam et al. 2004) and to *U. anastrephae* (Aluja et al. 2013). It has also been suggested that *D. longicaudata*, given its larger ovipositor,

could cause a local extinction by displacing *D. areolatus* when deprived of free space left by its competitors, as it has been suggested to explain the reduction of the dispersion range in Florida, USA, in the presence of *D. longicaudata* (Sivinski et al. 1998). For *U. anastrephae*, it has been suggested that the historic sympatry of *D. areolatus* and *U. anastrephae* depends on the ability of *D. areolatus* to avoid competition with the intrinsically superior competitor by exploiting hosts in larger fruits that are out of the reach of the smaller ovipositor of *U. anastrephae* (Sivinski et al. 1997, Aluja et al. 2013). However, our new findings suggest that both hypotheses can be reformulated in relation to the biology and oviposition behavior of *D. areolatus*.

An early action of *D. areolatus* against immature *A. obliqua* could represent an ecological advantage that prevents its displacement or local extinction by other competitors such as *U. anastrephae* and *D. longicaudata*, since these latter species invariably will attack mature larval stages that could already be parasitized by *D. areolatus*. According to Wang et al. (2003, 2008), in *F. arisanus* this earlier attack increases the probability to suppress the invasive larva through starvation or suffocation mechanisms. Field observations seem to support this assertion. Even though *D. longicaudata* has become established in numerous sites in Mexico where it has been released, its presence in *Spondias* species is inferior to that of *D. areolatus* (López et al. 1999, Sivinski et al. 2000, Montoya et al. unpublished data). Our data showed that this species also competes successfully against *U. anastrephae* by parasitizing eggs and recently hatched larvae, which enables *D. areolatus* to become the dominant parasitoid species in these hosts.

Laboratory studies have reported that *D. areolatus* is an inferior competitor relative to *D. longicaudata* and *U. anastrephae* (Paranhos et al. 2013, Aluja et al. 2013) because larvae of *D. longicaudata* and *U. anastrephae* kill larvae of *D. areolatus* during competition through sequential exposures. However, these studies were conducted using mature host larvae (3rd instar), which, according to our results, presents a disadvantage to *D. areolatus*.

One difficulty in rearing *D. areolatus* has apparently been the oviposition stimuli in oviposition units (artificial devices with third-instar host larvae mixed with food) (Eitam et al. 2003), which has not been a problem in the case of *D. longicaudata* because this species detects its host by larval vibrations when feeding or moving (Lawrence 1981). However, in light of our new findings, new perspectives on laboratory rearing of this species are realized. The use of late-stage eggs or neonate larvae, similar to *Fopius arisanus* rearing (Harris et al. 1991, Zenil et al. 2004, Rousse et al. 2005, Montoya et al. 2009), should be tested.

Unsuccessful attempts have been made to rear *D. areolatus* using fruits with third instar host larvae (Eitam et al. 2003). This could be because *D. areolatus* females need different stimuli, such as chemical signals emitted by host (eggs or young larvae) interacting with fruit volatiles, as has been demonstrated for *F. arisanus* (Rousse et al. 2007, Pérez et al. 2013).

The preimaginal development of *D. areolatus* in *A. obliqua* required approximately 27 days, and 7 preimaginal stages were classified: egg, three larval stages, prepupa, pupa, and pharate adult. The morphological observations of the preimaginal stages of

D. areolatus are in agreement with what has been reported for *F. arisanus* and *D. longicaudata* (Rousse et al. 2005, Carabajal-Paladino et al. 2010). However, the development of *D. areolatus* is more akin to *F. arisanus* because both exhibit a facultative synchronization between their larval development and that of its host, which is reflected in the long period of time for the first-instar larva, which finally changes when its host reaches the prepupal stage (Rousse et al. 2005). This type of development allows the second or third instars of *F. arisanus* (and probably *D. areolatus*) to occupy most of the available space inside the pupa, which facilitate the elimination of competing larvae (Wang et al. 2003, 2008).

Our study shows that *D. areolatus* can parasitize *A. obliqua* eggs and recently hatched larvae, giving an advantage over other parasitoids that attack the later-stage larvae. This finding represents a novel report regarding the oviposition behavior of this species, suggesting that it may occupy an ecological niche that was previously thought empty in the Americas. These findings also open new perspectives for the biological control of fruit flies. If mass rearing methods are developed, this will allow release of the most dominant fruit fly parasitoid species in the Neotropics.

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