Limits to the host range of the highly polyphagous tephritid fruit fly *Anastrepha ludens* in its natural habitat

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Abstract

Anastepha ludens (Diptera: Tephritidae) is a highly polyphagous fruit fly that is able to develop in a wide range of hosts. Understanding the limits of this pest's host range could provide valuable information for pest management and plant breeding for pest resistance. Previous studies have shown that guavas (*Psidium guajava* (Myrtaceae) L.), are not attacked under natural conditions by *A. ludens*. To understand this phenomenon, guavas were exposed to natural infestation by *A. ludens* and to other fruit fly species that infest guavas in nature (*Anastrepha striata* Schiner, *Anastepha fraterculus* (Wiedemann), *Anastepha obliqua* (Macquart)). Once the susceptible phenological stage of guavas was determined, fruit infestation levels were compared between *A. ludens* and *A. striata*. Choice and non-choice tests were performed under field-cage conditions. Under field conditions, guavas were susceptible to *A. striata* and *A. fraterculus* attack all the way from when fruit was undeveloped to when fruit began to ripen. No infestation by *A. ludens* was recorded under natural conditions. Similar results were obtained when forced exposures were performed, indicating that unripe guavas were preferred by *A. striata* over ripe fruit, and that infestation rates were higher at early fruit maturity stages. Under forced oviposition conditions, *A. ludens* larvae were unable to develop in unripe guavas but did so in fully ripe fruit. However, *A. ludens* fitness parameters were dramatically affected, exhibiting reduced survival and reduced pupal weight compared to conspecifics that developed in a natural host, grapefruit. We confirm that *P. guajava* should not be treated as a natural host of this pestiferous species, and suggest that both behavioral aspects and the fact that larvae are unable to adequately develop in this fruit, indeed represent clear limits to *A. ludens*'s broad host range.

Keywords: polyphagy, host range, host status, Tephritidae, *Anastrepha ludens*, *Anastrepha striata*, *Psidium guajava*, orchard management

Introduction

Host-plant use has been addressed from different perspectives including how plant chemistry (Ehrlich & Raven, 1964; Bernays & Chapman, 1994; Awmack & Leather, 2002) and parasite or predator pressure determine host range breadth (Bernays & Chapman, 1994; Stamp, 2001; Singer *et al.*, 2004). In the case of tephritid fruit flies, understanding host use is an essential tool for host status determination when commercial strategic decisions are taken to prevent the introduction of quarantine pests to countries or regions where they do not already occur. Aluja & Mangan (2008) describe methodological processes to unequivocally assign host status and point out that the use of host resistance (Aluja *et al.*, 2003; Papachristos *et al.*, 2008; Birke & Aluja, 2011), cultivar selection (Jalalulddin & Sadakathulla, 1999; Burrack & Zalom, 2008; Papachristos *et al.*, 2008; Guillén *et al.*, 2011; Aluja *et al.*, 2014*a*, *b*), ripening stage (Liquido *et al.*, 1989; Armstrong, 2001), and growing

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periods (Messina & Jones, 1990; Thomas, 2004), are viable alternatives to classical quarantine treatments.

Host acceptance and offspring performance are influenced by both physical (color, fruit firmness, epicuticular waxes, trichomes, resin and latex channels) and chemical traits (volatiles, free sugars, primary and secondary metabolites), which differ at each host fruit development and maturity stage (Aluja & Mangan, 2008) and are also modulated by factors that are inherent to the insect. These factors promote a so called 'motivational threshold' and are regulated by the insect's physiological stage (e.g., egg load, age) (Courtney *et al.*, 1989). Although in many cases the motivational threshold is high and non-hosts are accepted, plant chemistry of non-host species can prevent the successful development of the immature stages (Aluja *et al.*, 2014*b*). Predictive models which incorporate all of these aspects may help explain under which conditions certain fruits or vegetables are susceptible or remain resistant to polyphagous fruit flies, such as the Mexican fruit fly *Anastrepha ludens* Loew (Diptera: Tephritidae).

Comparative studies on specialist and generalist species of *Bactrocera* (Tephritidae) have concluded that the ability to expand their host range was influenced principally by adult female behavior (location, recognition and acceptance of a new host), and by female physiology (egg load, oviposition drive) (Fitt, 1986*a*), rather than by the ability of larvae to metabolize a novel nutritional composition or to overcome secondary metabolites (Fitt, 1986*b*; Aluja & Mangan, 2008).

This phenomenon (acceptance of a novel host) was also observed for some species of *Anastrepha*. For example, 'Manzano' peppers were unexpectedly found to be attacked by the Mexican fruit fly, *A. ludens* (Thomas, 2004). In this case, the availability of peaches was significantly reduced by drought and *A. ludens* females were forced to switch to a host that had never been reported before. Although the chemistry of this pepper differs widely from that of hosts usually exploited by *A. ludens*, peppers were accepted; volatiles did not appear to deter females and larvae developed well in the presence of high levels of alkaloids (Thomas, 2004). This is evidence that the 'innate' larval diet breadth for polyphagous species could be wider than the range of plants that are acceptable as oviposition substrates when the main preferred hosts are absent (Fitt, 1986*b*; Gratton & Welter, 1998; Aluja & Mangan, 2008).

A similar case is represented by guavas which are used by several fruit fly species as a main host or as an alternative host when preferred fruit species are not available (Birke & Aluja, 2011). Guavas are the preferred hosts of *Anastrepha striata*, *Anastepha fraterculus*, *Anastepha suspensa* (Loew) and *Ceratitis capitata* (Wiedemann) in the USA, but an occasional host of *Anastepha obliqua* (Sivinski *et al.*, 1997, 2004; Birke & Aluja, 2011; Costa *et al.*, 2011). Although *Psidium guajava* 'criollo' was long ago recorded as a host for *A. ludens* (Baker, 1944), recent evidence indicates that this tropical fruit is not naturally infested by *A. ludens* in Mexico (Birke & Aluja, 2011). It is surprising that *A. ludens* females fail to exploit guavas in nature, considering that their natural hosts (e.g., *Citrus* spp., *Casimiroa edulis* (La Llave & Lex.), mangos, peaches), occupy broadly overlapping ecological niches and often grow next to guava trees. The latter, contrasts with the case of *A. fraterculus*, another highly polyphagous fruit fly that belongs to the same species group as *A. ludens* (*fraterculus* species group (Norrbom, 2003)), which is able to thrive in guavas (Sivinski *et al.*, 1997, 2004; Birke & Aluja, 2011).

The aim of this study was to experimentally determine if there are clear-cut limits to the broad host range of *A. ludens* using guava as a model and by comparing its development with tephritid species that naturally infest guavas such as *A. striata*. We also assessed the host status of guava with respect to *A. ludens* following the host–status–determination–guidelines proposed by Aluja & Mangan (2008).

Materials and methods

Biological material

A. striata pupae stemmed from field-infested guavas collected in Jamapa, Veracruz, Mexico (19°02′N latitude, 96°11′W longitude, at 170 m above sea level (asl)). *A. ludens* pupae were obtained from flies reared for three generations on grapefruit in the laboratory after collection of field-infested *Citrus aurantium* L. from Miradores, Veracruz, Mexico (19°29′N latitude, 96°46′W longitude, at 920 m asl). All infested fruit were placed in baskets containing moist vermiculite as a pupation substrate. At emergence, adults were held in Plexiglas cages (30 × 30 × 30 cm^3) and offered *ad libitum* a mixture 3:1 of sugar (COSTCO®, Mexico), protein hydrolystate (Greif Bros. Corp., Delaware, OH, USA) and a water source. Environmental conditions in the laboratory were maintained at 26 ± 1°C, 65 ± 5% RH and LD 12:12 h photoperiod provided by 36 Watt Philips® daylight fluorescent bulbs.

Study sites

The experimental protocol was designed following methods outlined in Aluja & Mangan (2008). To guarantee that all environmental conditions where guava grows were represented in our study, we carried out experiments during the 2006/2007 fruiting seasons along an altitudinal gradient across three regions located at different elevations: San Julián (19°15′N latitude and 96°16′W longitude, at 26 m asl), Santa Fé (19°12′N latitude and 96°16′W longitude, at 36 m asl), Municipality of Veracruz; Rancho Viejo, Municipality of Emiliano Zapata (19°26′N latitude and 96°46′W longitude, at 912 m asl) and in a lot close to the Xico-Teocelo road junction, Municipality of Xico (19°23′N latitude, 96°57′W longitude, at 1137 m asl), Veracruz State, Mexico.

Fruit phenology and fruit characters

To standardize fruit phenological stages, we prepared a standard table based on fruit development, using conventional nomenclature originally proposed by Fleckinger (1945) and marbles as size references when possible. Visual assessment of pigmentation was also considered when guavas were fully developed. Based on Fleckinger (1945), fruit was categorized into one of seven developmental stages: flower (a flower having recently lost its petals), match (ovary enlargement), marble-fancy (15–20 mm ø), player (24–28 mm ø), mature green (full-sized fruit 100% green), turning yellow (full-sized fruit 80% green and 20% yellow) and completely mature (fig. 1).

Guava characteristics (fruit firmness, weight and sugar content)

Ten fruit from each of five trees at each study site (three locations) and at each selected developmental stage were harvested randomly during 2006 and 2007 (750 fruit/2006, 600 fruit/2007) and transported to the laboratory where physical characteristics (weight and firmness) and sugar content were measured a few hours after fruit harvest. Fruit were selected for uniformity following the standard table described above (fig. 1).

Phenological Stage	Picture	Size
STAGE 1. **Flower without petals/** **Petal fall (G*/67**)**		3-5 mm
STAGE 2. **Match/fruit setting** **(H1*/69)**		5-8 mm
STAGE 3. **Fancy marble size** **(I*/71**)**		15-20 mm
STAGE 4. **Player marble size/** **80% final size (I1*/73**)**		24-28 mm
STAGE 5. **Fully developed fruit** **(I3*/73**)**		> 32 mm
STAGE 6. **Turning yellow/fruit color** **changing (J*/81**)**		> 32 mm
STAGE 6. **Completely mature**		> 32 mm

Nomenclature of *Fleckinger code (Fleckinger 1945) and **BBCH code

Fig. 1. Diagram of fruit phenological stages used for natural and artificial exposure to *A. ludens* at three localities (San Julián (altitude 20–26 m), Emiliano Zapata (altitude 780 m) and road junction Xico-Teocelo (altitude 1130 m)). 1 = flower, 2 = match, 3 = fancy-size marble (18 mm Ø), 4 = player-size marble (24 mm Ø), 5 = mature green (28 mm Ø), 6 = turning-yellow, 7 = fully ripe.

Fruit resistance to puncture

Fruit firmness, was determined using a penetrometer with a 1 mm flat-tip, metal probe (four equatorial punctures per fruit) connected to a force gauge (Accuforce gauge III, model AF3010CE, Ametek, Mansfield and Green Division, Largo, FL, USA) on a motorized test stand (model 4665, Ametek, Mansfield and Green Division, Largo, FL, USA).

Fruit weight

Fruit was weighed using a standard electronic digital precision scale (Ohaus, TP4KD, USA).

Sugar content

Sugar content was measured by squeezing a piece of guava to obtain a drop of juice which was analyzed by means of a hand held-refractometer (ATAGO, PAL-1, Tokyo, Japan).

Comparison of natural and forced infestation rates

Experimental setup

Five trees in each of three locations (Santa Fé, Veracruz; Rancho Viejo, Emiliano Zapata and Xico-Teocelo road junction, Veracruz, Mexico) were selected to assess at which phenological/maturity stage guava fruit became susceptible

Table 1. Mean weight and mean fruit firmness at five development stages (1 = flower, 2 = match, 3 = fancy-size marble (18 mm Ø_, 4 = player-size marble (24 mm Ø), 6 = mature green (28 mm Ø)) at three localities (San Julián (20 m asl), Emiliano Zapata (780 m asl) and road junction Xico-Teocelo (1130 m asl), Veracruz).

Locality	Weight (gr)				
	Stage 1	Stage 2	Stage 3	Stage 4	Stage 6
San Julián, Veracruz	0.2 ± 0.02a	0 ± 0a	3.03 ± 0.13b	8.91 ± 0.27c	23.99 ± 0.87d
Rancho Viejo, Emiliano Zapata	0.42 ± 0.05a	0.34 ± 0.03a	1.87 ± 0.16b	5.83 ± 0.45c	9.66 ± 0.82d
Road junction Teocelo-Xico, Xico	0.15 ± 0.01a	0.71 ± 0.04b	2.732 ± 0.1c	6.84 ± 0.17d	11.68 ± 0.47e
	Peel firmness (Newtons)				
	Stage 1	Stage 2	Stage 3	Stage 4	Stage 6
San Julián, Veracruz	–	4.72 ± 0a	8.43 ± 0.57b	12.19 ± 0.31c	10.34 ± 0.44d
Rancho Viejo, Emiliano Zapata	–	6.89 ± 0.2a	7.18 ± 0.69b	10.88 ± 0.93c	8.18 ± 0.84d
Road junction Teocelo-Xico, Xico	4.02 ± 0.17a	6.32 ± 0.16b	7.83 ± 0.2c	13.76 ± 0.69d	6.56 ± 0.27e

Means followed by the same letters do not differ by the Tukey test at 5% probability.

to the attack of (a) naturally occurring fruit flies and (b) *A. ludens* and *A. striata* females under forced conditions. Forced infestations involved releasing gravid *A. ludens* and *A. striata* females onto (i) branches enclosed within a mesh bag (enclosed branches) or (ii) onto trees within field cages (enclosed trees) (see images in Aluja & Mangan, 2008).

Natural infestation test

In each of the three regions described above, six branches per tree holding 20–30 guava flowers were covered with mesh cloth to preclude any oviposition activity by wild flies and then later uncovered at each of five development stages to be exposed to natural fruit fly populations. Developmental stages assessed for this test were as follows: (1) flower without petals, (2) match, (3) fancy marble size, (4) 80% final size and (5) mature green fruit (fig. 1). After an 8-days exposure period branches were enclosed again to preclude any additional oviposition activity by wild flies and fruit was allowed to complete development. Finally, once fruit reached full maturity, guavas were harvested and transported to the laboratory in Xalapa, Veracruz. Picked fruit was placed individually in 250 ml plastic cups with moist vermiculite. Fruit was maintained in cups until complete decomposition allowing for full larval development and exit for pupation. Pupae were recovered and maintained in vermiculite in 250 ml plastic containers until adults emerged.

Forced infestation assays

Once the developmental stage at which guavas became susceptible to fruit fly attack had been determined, forced infestation trials were performed. The methodology was the same as the one described above for natural infestation. Branches were also covered with a cloth mesh but were artificially exposed to gravid *A. striata* and *A. ludens* females at a 1:1 (female/fruit) ratio (Aluja & Mangan, 2008). Stages used were: (4) 80% final size fruit, (5) mature green fruit and (6) mature yellowing fruit (fig. 1). Enclosures were examined every 48 h and dead females were replaced with flies of exactly the same age and condition. After 8 days, all females in each enclosure were recovered, transported to the laboratory, and held in 1 liter plastic cages. To ensure that exposed females were able to lay viable eggs, they were offered grapefruit as a substrate for 48 h; grapefruits were subsequently examined for evidence of larval infestation.

Guavas at the field sites remained on the tree until maturity and were then harvested and transported to the laboratory. Each fruit was also placed individually in 250 ml plastic cups, pupae were recovered, weighed and maintained in vermiculite in 250 ml plastic containers until adults emerged, whereupon date and sex ratio were determined at day one after emergence.

Comparison of A. striata and A. ludens *foraging behavior and host selection under choice and no choice conditions*

Choice and no choice tests were performed to determine differences in foraging behavior and host selection of gravid *A. striata* and *A. ludens* females under field-cage conditions. Tests were performed to ascertain if non-hosts for *A. ludens* (guava) or *A. striata* (grapefruit) were accepted for oviposition by both *A. striata* and *A. ludens*. Three mid-sized guava trees were selected at the high elevation site (Xico-Teocelo road junction) and each tree was enclosed using a 4 × 4 × 4 m³ mesh field cage (BioQuip Products, Compton CA). For each tree we selected 120 fully developed guavas (stage 5, fully sized) which were covered with white paper bags. At each observation day during the 14-days trial, 20 bags per tree were removed and 20 gravid females (*A. striata* or *A. ludens* at a 1:1 female: fruit ratio) were released. 'Marsh' grapefruits (120 fruits) were obtained from experimental trees in Alborada, Veracruz (15.3 km from the experimental area). Fruits were selected and were hung next to each guava using hemp thread. Treatment combinations for fruit fly species (*A. ludens* or *A. striata*), and host or non-host, under choice or non-choice situations, were set and rotated daily (table 1). Tests were replicated four times, each time using a new naïve cohort of flies. Observations took place from 09:00 to 15:00 h, and were performed by one observer per tree using a scan observation method (one scan per 15 min) (Aluja & Birke, 1993). The following parameters were recorded: visits (host or non-host), oviposition attempts (probing that was not followed by ovipositor dragging), oviposition and duration of oviposition. After the 6 h observation period bags were discarded to prevent reuse.

Effect of guava development stage on host-selection by A. ludens *and* A. striata

To establish whether guava developmental stage influenced foraging behavior and offspring performance, an

Fig. 2. Photograph showing guava racemes at four maturity stages. Guavas were exposed to *A. striata* and *A. ludens* in field-cage tests.

additional trial was set up. One guava tree which harboured several guava clusters in single branches (fig. 2) was enclosed within a $4 \times 4 \times 4$ m^3 field-cage. Each guava cluster had fruit of each of four maturity stages: (a) green undeveloped fruit (stage 4), (b) mature green (stage 5), (c) turning yellow (stage 6) and (d) fully ripe fruit (stage 7) (fig. 2). A total of 80 clusters (640 guavas) were enclosed within white paper bags. All fruit bags were labeled according to fruit stage and fruits were numbered with a non-toxic permanent marker (Sharpie, NewellRubbermaid Company, Shelbyville, TN, USA).

As in the first trial, observations began at 09:00 h and ended at 15:00 h. A total of 80 fruits (20 clusters) were exposed by releasing 20 *A. ludens* or 20 *A. striata* females into each cage on each day of the experiment. The species of fly released was alternated each day and a scan observation method was followed (Opp & Prokopy, 1986). The same methodology was used as in the previous trial described above, and the same foraging and oviposition activities were recorded. Tests were replicated four times for each fruit fly species (8 days observation trial) and all fruit was harvested after 15 days. Fruit was transported to the laboratory and infestation level was assessed once fruit decomposed using the same procedure described previously.

Data analyses

Statistical analyses were performed using STATISTICA Version 11 (Statsoft, 1998). Prior to parametric tests, all data were checked for normality and homogeneity of variance (Shapiro–Wilk's test). If these assumptions were violated data were transformed. Data on skin firmness, fruit weight and sugar content (2006–2007 and 2007–2008) were subjected to an analysis of variance (ANOVA) with a hierarchical structure nesting fruit on tree and using a generalized linear model. Infestation levels were in all cases calculated as the number of adults obtained per fruit and were normalized by rank transformation (Conover & Iman, 1981) and also subjected to an ANOVA with a hierarchical structure nesting fruit on tree. Mean number of oviposited fruit at different maturity stages were subjected to the same analyses. The possible association between fruit maturity/firmness and infestation level were examined using Pearson's correlation procedure. Pupal weight

was compared for *A. ludens* and *A. striata* among maturity stages by means of a one-way ANOVA. *Post-hoc* Tukey tests were performed when necessary. Choice and no-choice activity patterns (number of fruit visits, oviposition attempts and successful oviposition events) were analyzed using Mann–Whitney or Kruskal–Wallis tests.

Results

Fruit characteristics at different fruiting stages

Peel firmness varied significantly among guava maturity stages and location during season 2007 but did not for location in the season 2006 (2007: $F = 138.33$, df = 4,573, $P < 0.0001$ maturity; $F = 4.275$, df = 2,573, $P < 0.014$ location) and (2006: $F = 350.8$, df = 2,438, $P < 0.0001$ maturity; $F = 2.22$, df = 2,438, $P < 0.11$ location) (tables 1 and 2, fig. 1). Fruit firmness reached its highest peak shortly before fruit started to significantly enlarge (stage 4) and decreased when fruit began to mature (tables 1 and 2). Fruit weight exhibited significant differences among fruit maturity stages and locations during 2007 but did not for location during the 2006 season (2007: $F = 364.67$, df = 4,583, $P < 0.0001$ maturity; $F = 91.42$, df = 2,583, $P < 0.0001$ location), (2006: $F = 177.13$, df = 3,440, $P < 0.0001$ maturity; $F = 1.24$, df = 2,440, $P < 0.0001$ location) (tables 1 and 2, fig. 1). With respect to sugar content, a highly significant difference among fruit maturity stages and locations was also found (2006: $F = 590.39$, df = 3,440, $P < 0.0001$ maturity; $F = 10.14$, df = 2, 440, $P = 0.0001$, location) (table 2).

Assessment of fruit infestation under natural conditions

Infestation levels of 540 naturally exposed fruit revealed that guavas are only infested by *A. striata* (84%) and *A. fraterculus* (16%) in our study region. Guavas were susceptible to *A. striata* and *A. fraterculus* attack once fruit reached ca. 80% of its total size (table 3). Importantly, under natural conditions neither *A. ludens* larvae nor adults were recovered from naturally exposed fruit (table 3).

Assessment of fruit infestation rates by comparing A. ludens and A. striata under forced conditions

Our experiment, under forced conditions, exposing undeveloped, mature green and turning-yellow guavas, revealed highly significant differences in infestation rates between both fruit fly species and among fruit maturity and locations (nested ANOVA, $F = 99.4$, df = 1,690, $P < 0.001$ fly species; $F = 18.35$, df = 2,890, $P < 0.0001$ maturity; $F = 8.16$, df = 2,690, $P < 0.004$ location) (fig. 3). We found that guava maturity was negatively correlated with *A. striata* infestation ($r = -0.301$) and fruit firmness was positively correlated with infestation ($r = 0.303$). We obtained a total of 1531 *A. striata* pupae from undeveloped firm fruit, 684 from green mature fruit and 304 from soft turning-yellow guavas. In the case of *A. ludens*, we did not detect any correlation among fruit maturity stages, and infestation remained consistently low, compared with *A. striata* (fig. 3). Notably, in the case of the 335 guavas exposed to *A. ludens* females (1:1 female: fruit proportion), 44 (13% of the total) individuals died while attempting to insert their aculei (boring activity) into the fruit or while laying eggs.

Table 2. Mean weight, mean peel firmness and mean sugar content (Brix) of guava fruits at four development stages (4 = player-size marble (24 mm Ø), Stage 5 = mature green, Stage 6 = turning yellow and Stage 7 = mature) at three localities (Santa Fé (20 m asl), Emiliano Zapata (780 m asl) and road junction Xico-Teocelo (1130 m asl)).

Locality	Weight (g)			
	Stage 4	Stage 5	Stage 6	Stage 7
San Julián, Veracruz	9.82 ± 0.41	16.42 ± 0.72	25.55 ± 1.8	21.31 ± 1.41
Rancho Viejo, Emiliano Zapata	11.68 ± 0.47	12.81 ± 0.85	24.75 ± 0.98	27.93 ± 0.94
Xico-Teocelo road junction, Xico	11.37 ± 0.64	13.69 ± 0.37	23.7 ± 1.02	31.87 ± 1.56
	Peel firmness			
	Stage 4	Stage 5	Stage 6	Stage 7
San Julián, Veracruz	16.25 ± 2.14	9.11 ± 2.58	8.2 ± 2.9	5.52 ± 3.27
Rancho Viejo, Emiliano Zapata	14.81 ± 2.16	12.73 ± 2.34	8.34 ± 2.32	3.55 ± 2.41
Road junction Xico-Teocelo, Xico	13.57 ± 1.78	11.84 ± 1.88	6.16 ± 1.91	3.38 ± 1.94
	Sugar content			
	Stage 4	Stage 5	Stage 6	Stage 7
San Julián, Veracruz	3.18 ± 0.1	4.17 ± 0.45	10.90 ± 0.16	11.40 ± 0.3
Rancho Viejo, Emiliano Zapata	1.26 ± 0.15	8.35 ± 0.22	9.36 ± 0.1	9.64 ± 0.15
Road junction Xico-Teocelo, Xico	1.25 ± 0.07	7.87 ± 0.12	8.67 ± 0.15	8.74 ± 0.16

Table 3. Adults obtained from natural infested guavas at five development stages 1 = flower, 2 = match, 3 = fancy-size marble (18 mm Ø), 4 = player-size marble (24 mm Ø), 5 = mature green (28 mm Ø), 6 = turning-yellow and 7 = fully ripe fruit at three localities (Santa Fé (20 m asl), Emiliano Zapata (780 m asl) and Xico-Teocelo road junction (1130 m asl)).

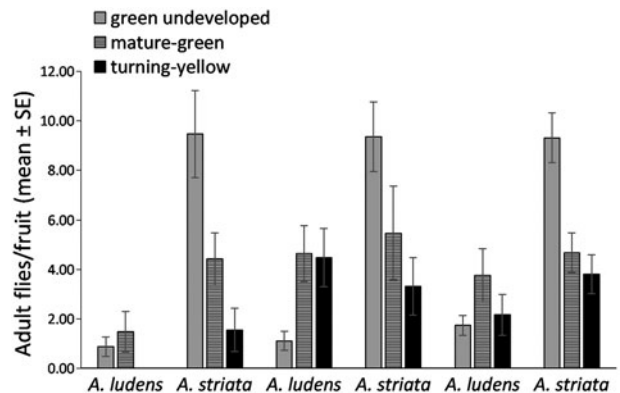| Locality	Development stage	Fly species		
		A. striata	*A. fraterculus*	*A. ludens*
San Julián	Stage 1	0	0	0
San Julián	Stage 2	0	0	0
San Julián	Stage 3	0	0	0
San Julián	Stage 4	0	0	0
San Julián	Stage 5	7 ♀ y 6 ♂	1 ♀ y 1 ♂	0
Rancho Viejo	Stage 1	0	0	0
Rancho Viejo	Stage 2	0	0	0
Rancho Viejo	Stage 3	0	0	0
Rancho Viejo	Stage 4	7 ♀ y 2 ♂	0	0
Rancho Viejo	Stage 5	12 ♀ y 8 ♂	0	0
Xico-Teocelo road junction	Stage 1	0	0	0
Xico-Teocelo road junction	Stage 2	0	0	0
Xico-Teocelo road junction	Stage 3	0	0	0
Xico-Teocelo road junction	Stage 4	0	0	0
Xico-Teocelo road junction	Stage 5	0	2 ♀ y 2 ♂	0

Fig. 3. Adults obtained from guavas (mean ± SE) exposed to forced infestations by *A. striata* and *A. ludens* at three maturity stages (4 = 24 mm Ø, 6 = mature green 28 mm Ø, 7 = turning-yellow) at three localities: San Julián/Santa Fé (altitude 20–26 m), Emiliano Zapata (altitude 780 m) and road junction Xico-Teocelo (altitude 1130 m).

Effect of guava on pupal weight

A. ludens pupae obtained from guavas (9.24 ± 0.56 mg) weighed less than a half of pupae obtained from the natural host grapefruit (21.42 ± 0.94 mg). In addition, we found that pupal weight varied significantly among maturity stages in the case of both fruit fly species (one-way ANOVA, $F = 3.66$, df = 2, 447, $P = 0.03$ *A. ludens*; $F = 4.38$, df = 2, 1003, $P = 0.013$ *A. striata*) (fig. 4).

Foraging behavior and host selection trials comparing *A. striata* and *A. ludens* using choice and no choice tests

Choice test

When given a choice (guava and grapefruit), *A. ludens* females visited significantly more grapefruits than guavas (Mann–Whitney, $Z = -1.18$, $P < 0.6$) and oviposited significantly more frequently in grapefruit than in guava (Mann–Whitney, $Z = -2.165$, $P < 0.03$). Overall, 64% of all grapefruit and 36% of all guavas used in the experiment were oviposited by *A. ludens* females. In contrast, *A. striata* females never oviposited or attempted to oviposit in grapefruits doing so only in guavas (fig. 5A).

No-choice test

When only guavas or grapefruits were offered, *A. ludens* and *A. striata* females mainly visited their preferred hosts
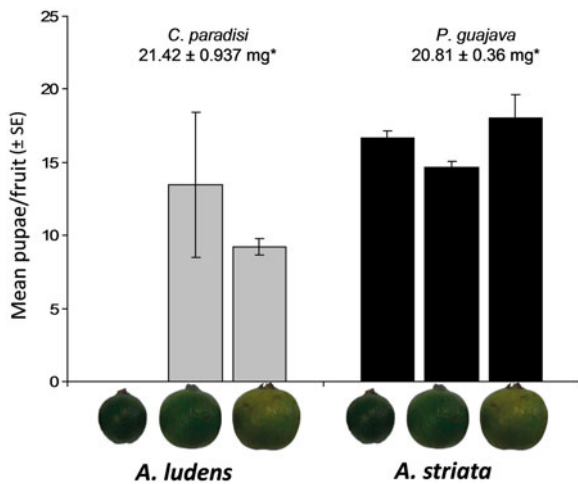
Fig. 4. *A. ludens* and *A. striata* pupal weight of insects that developed in guavas (mean ± SE) at three maturity stages (4 = 24 mm Ø, 6 = mature green, 28 mm Ø, 7 = turning-yellow at three localities San Julián/Santa Fé (altitude 20–26 m), Emiliano Zapata (altitude 780 m) and road junction Xico-Teocelo (altitude 1130 m).

(Kruskal–Wallis, Chi-square = 12.95, df = 3, $P < 0.001$) (fig. 5B). Under these no-choice conditions, *A. ludens* readily attempted or indeed oviposited into guavas, something not observed when females were given a choice between the natural host grapefruit and guavas. The total number of oviposition attempts per replicate varied significantly between both fruit fly species (Kruskal–Wallis, Chi-square = 8.89, df = 3, $P < 0.03$); *A. striata* attempted to oviposit significantly more frequently than *A. ludens* in guavas but, as was the case in the no-choice experiment, never did so in grapefruits (fig. 5B). The number of ovipositions followed by ovipositor dragging (i.e., effective oviposition) by *A. ludens* was significantly higher on grapefruit than on guava (Kruskal–Wallis, Chi-square = 15, df = 3, $P < 0.001$). Overall, 90% of grapefruits and only 10% of guavas were accepted as oviposition substrates by *A. ludens* females.

Effect of guava developmental stage on host-selection by A. ludens *and* A. striata

Guava developmental stage trials clearly showed that *A. ludens* and *A. striata* females oviposit into fruit at contrasting maturity stages. *A. ludens* preferred fully developed, yellowing or yellow mature guavas, whereas *A. striata* females preferred green undeveloped or fully developed green guavas as oviposition sites (nested ANOVA, $F = 7.22$, df = 3, 21, $P < 0.001$) (fig. 6A).

Infestation of guavas was significantly lower for *A. ludens* than for *A. striata* (nested ANOVA, $F = 4.88$, df = 3, 21, $P < 0.048$, fly specie) and fruit physiological stage significantly influenced adult recovery (nested ANOVA, $F = 4.41$, df = 3, 21, $P < 0.014$, fruit physiological stage) (fig. 6B).

Discussion

Although guavas have been believed to be natural hosts of *A. ludens* based on early observations of anecdotal nature (Baker *et al.*, 1944), our results following the rigorous guidelines set out by Aluja & Mangan (2008) demonstrate that *P. guajava* cv 'Criollo de Veracruz' is not a natural host for *A. ludens* and

should be treated for quarantine purposes as a conditional host. Results obtained using natural infestation trials performed along an elevation gradient, showed that only *A. striata* and *A. fraterculus* naturally attack guava, confirming earlier work by us (Birke & Aluja, 2011). Although in our study *A. fraterculus* was not abundant (only a few pupae were obtained), Sivinski *et al.* (2004) clearly showed that *A. fraterculus* can indeed infest guavas in large numbers. These authors also showed that the populations of *A. fraterculus* and *A. striata* exhibit marked annual fluctuations in the same guava trees over time. Over a 6-year period, they showed that in some years one species is almost non-existent, while in others both show up in equal numbers. These results (i.e., Sivinski *et al.*, 2004) most likely explain the phenomenon we observed, with very few *A. fraterculus* individuals infesting guavas the year we ran our experiments, when in fact this fruit represents a commonly infested wild host throughout the range of the species' distribution. Extensive guava sampling elsewhere in the Mexican states of Veracruz, Chiapas, Morelos, Aguascalientes and Zacatecas have indicated that only *A. striata, A. fraterculus* and occasionally *A. obliqua*, can be recovered in nature from this host in Mexico (Aluja *et al.*, 1987, 1998; Aluja & Birke, 1993; Sivinski *et al.*, 1997, 2004; Padilla, 2002; Birke & Aluja, 2011).

When analyzing at which phenological stage guavas became susceptible to naturally occurring fruit flies, we found that unripe guavas and mature green guavas were almost invariably used by *A. striata* and *A. fraterculus*. Same results were recently published for *A. fraterculus* in Brasil (Bisognin *et al.*, 2015). Fruit firmness, sugar content was not related with infestation rates. Similarly, in some *Bactrocera* species susceptibility of guavas to *Bactrocera* infestation was not related to sugar content but was affected by fruit pH or total phenol content (Jalalulddin & Sadakathulla, 1999). Ripening of guava is associated with a decrease in guava firmness, astringency and less phenolic compounds in the peel (Da Silva *et al.*, 2000; Padilla, 2002; Bashir & Abu-Gouk, 2003; Pérez *et al.*, 2008). High total phenolic content also has been shown to reduce larvae infestation and increase apple resistance to *A. ludens* attack (Aluja *et al.*, 2014b).

Specialization of both fruit fly species (*A. striata* and *A. fraterculus*) in overcoming the secondary chemistry in unripe guavas may partially explain why *A. ludens* is not able to reproduce successfully in unripe guavas. Additionally, as reported here, 13% of all exposed *A. ludens* females died when attempting to oviposit into unripe, green guavas. We believe that the abrupt death of ovipositing *A. ludens* females may have been caused by exposure to volatiles which are known to be emitted by guava leaves or unripe fruit and have repellent or toxic effects on other insects (e.g., psyllids) (Zaka *et al.*, 2010) and possibly by unripe guavas when punctured by the female aculeus. Although we have to accept that the host used to originally rear *A. striata* and *A. ludens* may have in some way influenced guava preference, it does not explain the death of *A. ludens* females and the larval inability to develop successfully in guavas. As mean oviposition time into guavas for *A. ludens* is ∼10 times longer than the oviposition time by *A. striata* or *A. fraterculus*, the duration of exposure to volatiles could have been extended in *A. ludens*. Alternatively, *A. striata* and *A. fraterculus* females may not be susceptible to the toxins as they both have a long evolutionary history of development in guavas. On the other hand, when eggs are laid in yellowing or fully ripe fruit, larvae of *A. ludens* have only a small time-window in which to develop as guavas senesce and decompose rapidly. This partially explains why the few *A. ludens* pupae recovered from guavas
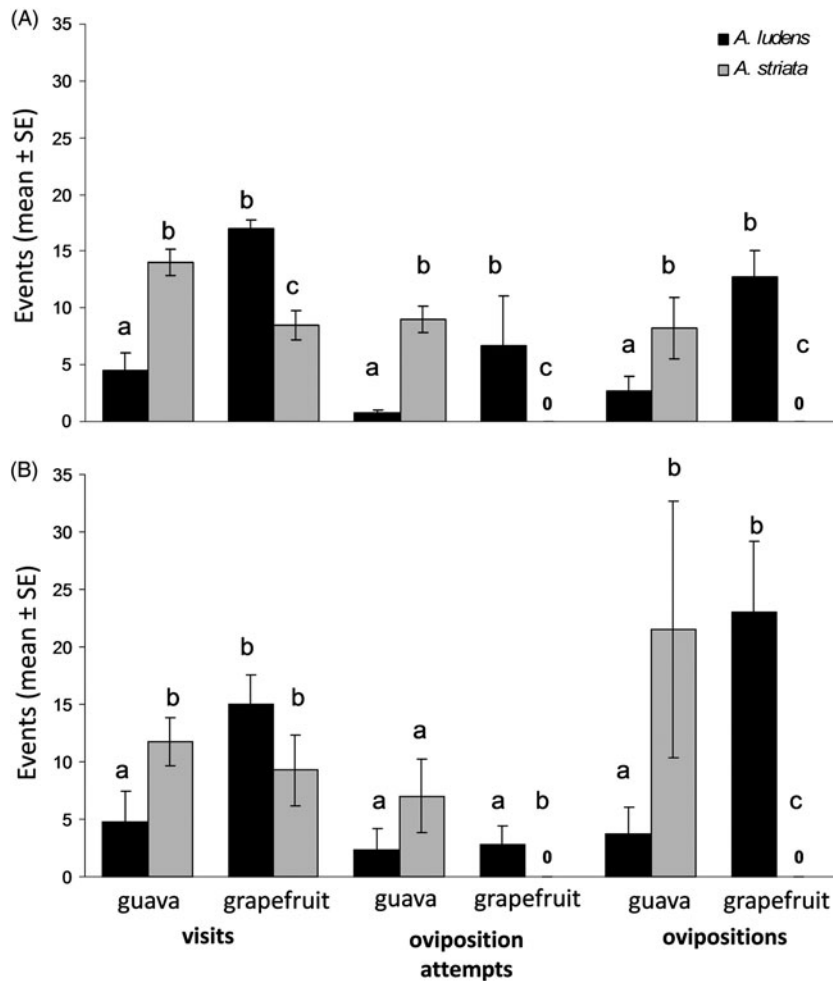
Fig. 5. (A) Visits, oviposition attempts and effective ovipositions (mean ± SE) by *A. ludens* and *A. striata* female under no choice and (B) Choice conditions.

were very small in size. Under natural conditions, ripe guavas fall to the ground and decompose rapidly, or are eaten by birds, mammals and other insects. Under these conditions, developing larvae would not be able to complete development and pupate. So, there appears to be a number of obstacles that *A. ludens* would need to overcome to successfully infest and complete its life cycle in guavas. Based on our previous studies and the results reported here, it seems that guavas represent a true limit to the extreme polyphagy that *A. ludens* exhibits.

Forced, comparative infestation trials (enclosed branches) also revealed that *A. striata* preferred unripe to fully developed green guavas, which was not the case for *A. ludens* and seems not to be the case for *A. fraterculus* populations in Brasil (De Oliveira *et al.*, 2015). *A. ludens* females preferred yellowing fruit (guavas) as reported previously (Berrigan *et al.*, 1988; Birke *et al.*, 2006) and only attempted to oviposit into green guavas when not given a choice. Moreover, *A. striata* never attempted to oviposit into grapefruits. As shown recently (Aluja *et al.*, 2011), *A. ludens* cannot reabsorb oocytes when deprived of suitable oviposition sites and can therefore be easily forced to lay eggs in almost any fruit (Aluja & Mangan, 2008). Guavas close to full maturity (yellow-turning stage) were accepted by *A. ludens* females for oviposition, as occurs in other fruits (e.g.,

mangoes and grapefruits) (Berrigan *et al.*, 1988; Aluja, 1993; Birke *et al.*, 2006) but turned out to be a poor host for larval development. We only obtained small numbers of adults from guavas when compared to those recovered from grapefruit.

Our results could have important practical implications for risk management and quarantine treatment requirements. First, we present robust data supporting the notion that guavas should not be treated as a natural host of *A. ludens*. Based on Aluja & Mangan (2008), and our own extensive field work (Birke & Aluja, 2011), we suggest that *P. guajava* cultivar 'Criollo Veracruz' should henceforth be treated as a conditional host of *A. ludens*. Even under artificial, forced-infestation conditions, *A. ludens* infestation levels were very low, a significant number of females died during the act of oviposition, and the few *A. ludens* adults that emerged were significantly smaller than adults stemming from the natural host grapefruit. Further studies should focus on comparing potential differences in infestation among commercially grown cultivars of guava, as our assays were only performed using wild cultivars growing in Veracruz, Mexico. This is particularly relevant given that fruit characteristics differ markedly among cultivars, and wild fruit or traditional cultivars tend to maintain natural resistance gene sets which impede insect attack (Jalalulddin & Sadakathulla,
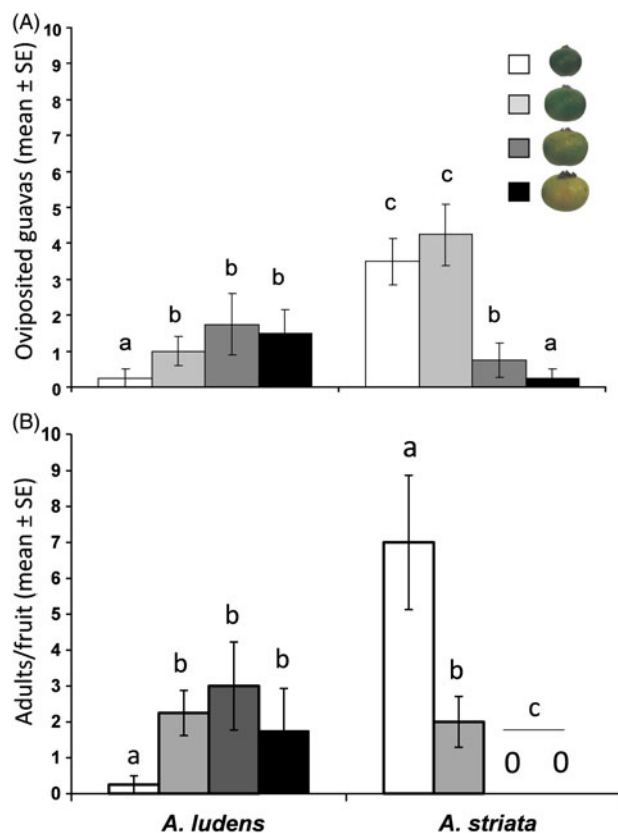
Fig. 6. (A) Number of ovipositions (B) Infestation level (pupae/fruit) (mean ± SE) in each of four guava maturity stages when exposed to *A. ludens* and *A. striata* females in field-cage tests.

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1999; Rodriguez-Saona *et al.*, 2011; Aluja *et al.*, 2014*a*, *b*; De Oliveira *et al.*, 2015). Additionally, we believe that late insecticide sprays in commercial guava orchards are redundant as fruit at this stage are already infested with *A. striata* and *A. fraterculus* given that, as shown here, guavas become susceptible to infestation to these two species when they are unripe.

Finally, we concur with Fitt (1986*a*) who concluded that limitations in polyphagous fruit fly species exploiting new hosts is a complex, multifactorial phenomenon that is mainly constrained by behavioral aspects and not by larval inability to develop in a novel substrate. This may change if the female motivational threshold is high (e.g., high egg load combined with low preferential host availability) (Courtney *et al.*, 1989). Fitt (1986*a*, *b*) compared several *Bactrocera* species (*B. tryoni*, *B. jarvisi*, *B. cucumis*, *B. musae* and *B. cacuminatus*) which differ in host range and observed that larvae of most species could develop in different types of fruit under laboratory conditions, but did not use these hosts in nature. In contrast to Fitt's work (1986*a*), our results also suggest that limits exist to the extremely large host range of *A. ludens*, as immatures developed very poorly or not at all in guavas. This had not been reported previously for fruit flies as all nutritional studies using immatures (larva) have been performed in the laboratory using harvested fruit which tends to lose certain chemical characteristics after having been harvested (Fitt, 1986*a*; Leyva *et al.*, 1991). We therefore believe to have identified an excellent model system to gain further insight into the reasons guavas represent such a major barrier to the reproduction of this insect.

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