

# Precopulatory mating and postzygotic isolation between two walnut-infesting species of *Rhagoletis* from Mexican highlands

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## Abstract

Pleistocene glacial and postglacial cycles producing contraction and expansion of temperate habitats have resulted in substantial diversification among several plant and animal taxa of Nearctic origin undergoing periods of isolation and secondary contact in high-elevation areas of Mexico. One of such groups are walnut-infesting fruit flies in the genus *Rhagoletis* (Diptera: Tephritidae), comprised of six recently derived species among which phylogenetic relationships have been difficult to unravel using conventional molecular methods. Here, we examined pre- and postzygotic isolation between two genetically similar and morphologically distinct species which are currently parapatric in central Mexico. Local adaptation driven by differences in host plant phenology between *Rhagoletis zoqui* Bush and *Rhagoletis ramosae* Hernández-Ortiz resulted in allochronic isolation. Despite the existence of precopulatory mating isolation, there was a substantial number of hybrid matings in field cages where conspecific and heterospecific males and females were simultaneously released. The bulk of mating activity took place on host fruit. *Rhagoletis zoqui* females were more reluctant to mate with *R. ramosae* males than with males of their own species. Distinctive behavioral differences were observed between males during contests, fruit guarding, and approach to conspecifics on fruit. There was also some asymmetric postzygotic isolation, with the hybrid combination of *R. zoqui* males and *R. ramosae* females resulting in lower egg hatch than other mating combinations. Results were consistent with those of a phylogenetic study suggesting recent divergence of *R. ramosae* from *Rhagoletis completa* Cresson and *R. zoqui* in the Sierra Madre Oriental and the Mexican Trans Volcanic Belt.

## Introduction

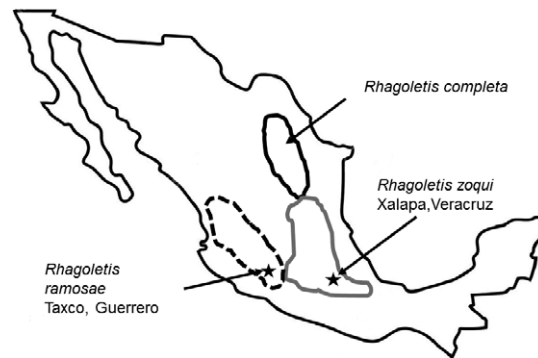
Historical climatic variation producing pulses of contraction and expansion of particular habitats and periods of isolation and secondary contact has been found to play an important role in divergence, genetic structure, and distribution of many Neotropical species (Avice, 2000; Hewitt, 2000, 2004; Feder et al., 2005). In particular, Pleistocene glacial and interglacial cycles have played a key role in producing some of the current species diversity patterns (Huntley & Webb, 1989; Joseph et al., 1995; Hewitt, 1996;

Roy et al., 1996; Willis & Whittaker, 2000). During glacial cycles, species with affinity for temperate climates were displaced to latitudes where less extreme climatic conditions allowed survival (Hewitt, 2004; Solis et al., 2006; Provan & Bennett, 2008). Following glaciations, such species took refuge in high-elevation areas surrounded by dry and warm lowland habitats (Haffer, 1969; Burnham & Graham, 1999; Knowles, 2001; Hooghiemstra & van der Hammen, 2004; Bush & de Oliveira, 2006). Isolation in refugia and genetic drift triggered differentiation in ecological islands that could be reinforced or lost during periods of secondary contact (Fosberg, 1983; Petit et al., 2003; Zarza et al., 2008; De Mello, 2011; Ramírez-Barahona & Eguiarte, 2013). Such patterns have resulted in current diversity patterns in many lineages (Knowles, 2001; Salomon, 2001; Shepard & Burbrink, 2009; Qu et al., 2011).

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Historically, the most prevalent mode of speciation was thought to be allopatric, wherein genetic differences would accumulate due to disruptive environmental selection and genetic drift in a state of geographic isolation (Mayr, 1963; Salomon, 2001). After secondary contact, according to vicariance theory, prezygotic isolation tends to evolve to prevent maladaptive hybridization (Dobzhansky, 1942). However, much evidence has been gathered supporting the likelihood and prevalence of speciation modes not involving periods of geographical isolation (Berlocher & Feder, 2002; Mallet, 2008). Additionally, genetic changes generating inherent barriers to gene flow between populations can evolve in geographic isolation, whereas others can arise in sympatry, resulting in a mixed or plurality divergence mode (Xie et al., 2007). The relative strength of pre- and postzygotic isolation may shed some light into the evolutionary history and divergence patterns (Coyne & Orr, 1989, 1997, 2004) and a fully resolved biogeography is necessary for the understanding of speciation within particular groups (Xie et al., 2007).

Many insect species currently inhabiting North America evolved in mountainous areas as a result of Pleistocene isolation and contact cycles (Ross, 1953; Howden, 1969). Mexico has a particularly intricate topography that produced a rich complex of temperate pine-oak ecological islands surrounded by warm dry habitats that underwent several contractions and expansions (Martin & Harrell, 1957; Howden, 1963, 1969). A good example of insect diversification in high-elevation islands are flies in the genus *Rhagoletis* (Feder et al., 2003, 2005; Michel et al., 2007; Xie et al., 2008), in particular those species comprised in the walnut-infesting *suavis* species group (Bush, 1966; Foote, 1981; Bush & Smith, 1998). The *R. suavis* species group is currently formed by five species whose phylogenetic relationships have been examined (*Rhagoletis suavis* Loew, *Rhagoletis completa* Cresson, *Rhagoletis zoqui* Bush, *Rhagoletis boycei* Cresson, and *Rhagoletis juglandis* Cresson) (Bush, 1966, 1968; Foote, 1981; Smith & Bush, 2000), and *Rhagoletis ramosae* Hernández-Ortiz, a more recently described species (Hernández-Ortiz, 1985). All species in the group, except *R. suavis*, occur in Mexico (Bush, 1966; Foote, 1981; Foote et al., 1993; Smith & Bush, 2000), whereas *R. zoqui* and *R. ramosae* are endemic to central Mexico (Bush, 1966; Hernández-Ortiz, 1985; Rull et al., 2013a). *Rhagoletis zoqui* is distributed in a zone encompassing mid-elevation areas (1 000–2 000 m) in Veracruz, Tlaxcala, Puebla, Hidalgo, and San Luis Potosí. *Rhagoletis ramosae*'s range encompasses Michoacán, Guerrero, Estado de México, Jalisco, and Nayarit (Bush, 1966; Foote, 1981; Hernández-Ortiz, 1985; Foote et al., 1993; Smith & Bush, 2000; Rull et al., 2013a,b) (Figure 1).



**Figure 1** Map showing the distribution of *Rhagoletis completa* (black line), *Rhagoletis zoqui* (grey line), and *Rhagoletis ramosae* (dashed line) in Mexico. Stars represent collection sites of infested fruit.

Molecular phylogenies on species in the *R. suavis* group have allowed to place *R. suavis* and *R. juglandis* in clearly delimited clades, grouping the remaining species in a tight unresolved cluster (Smith & Bush, 1997, 2000; Frey et al., 2013; Rull et al., 2013a). The relationship between *R. ramosae* and a group comprising *R. zoqui*, *R. completa*, and *R. boycei* was unclear (Rull et al., 2013a), until a recent study supported a clade in which *R. ramosae*, *R. zoqui*, and *R. completa* are closest relatives (Glover et al., 2018). Within this clade, Glover et al. (2018) found no mtDNA resolution between *R. completa* and *R. zoqui* suggesting gene flow and/or incomplete lineage sorting. A hybrid zone between *R. completa* and *R. zoqui* in Acahualtes, Mexico (Rull et al., 2012), supports the hypothesis of introgression. Regardless, the results indicated that *R. ramosae* is most closely related to *R. completa* and *R. zoqui* and not to other members the *R. suavis* group, implying that *R. ramosae* diverged relatively recently from *R. completa* and *R. zoqui* in the southern portion of the Sierra Madre Oriental (SMOr) and Eje Volcánico Trans Mexicano (EVTM) of Mexico.

In the course of an extensive sampling effort to establish the distribution of walnut-infesting *Rhagoletis* spp. in Mexico (Rull et al., 2013a), the discovery of natural hybrids between *R. zoqui* and *R. completa* led Rull et al. (2012) and Tadeo et al. (2013) to explore the evolution of pre- and postzygotic isolation between these two groups. Results of reproductive compatibility studies between these species revealed little precopulatory mating and postzygotic isolation. More recently, *R. ramosae* and *R. completa* were also found to be capable of hybridization in the laboratory; however, they displayed greater precopulatory mating and postzygotic isolation than *R. zoqui* and *R. completa* (E Tadeo, M Aluja & J Rull, unpubl.). The

geographical range of *R. zoqui* limits in the north with that of *R. completa* and to the west with *R. ramosae* (Rull et al., 2013a), suggesting that *R. zoqui* might have been a conduit for geneflow between *R. ramosae* and *R. completa*. Examination of the COI and COII genes revealed that *R. ramosae* differs from *R. zoqui* and *R. completa* only by two derived substitutions (Glover et al., 2018). Thus, *R. ramosae* in the EVTm diverged relatively recently from *R. completa* and *R. zoqui* in the SMO and should display both precopulatory mating (close to random) and postzygotic (with all cross types producing viable hybrids without distortion of sex ratio) reproductive compatibility with *R. zoqui*. Here, as a complement for recent phylogenetic studies aimed at understanding the origin of *R. ramosae*, we report on experiments exploring the existence and strength of allochronic (time from pupation to adult eclosion), precopulatory mating and postzygotic isolation between *R. ramosae* and *R. zoqui*.

## Materials and methods

### Source of flies

*Rhagoletis zoqui* was recovered from infested *Juglans pyriformis* Liebmann fruit (Juglandaceae), collected in Xalapa (19°30'45.81"N, 96°56'38.86"W, 1 342 m altitude) and Coacoatzintla Veracruz (19°38'48.95"N, 96°56'29.76"W, 1 440 m) between August and September 2011, whereas *R. ramosae* was obtained from *Juglans major* (Torr) Heller var. *glabrata* Manning collected in Taxco Guerrero (18°33'16.9"N, 99°39'31.9"W, 1 780 m). Fruit was taken to the laboratory at the Instituto de Ecología (INECOL), in Xalapa Veracruz, and processed following methods outlined in Rull et al. (2006) to recover pupae. Pupae were placed in 200-ml plastic cups lined at the bottom with a 2-cm vermiculite layer and humidified regularly with a sodium benzoate (C<sub>6</sub>H<sub>5</sub>COONa) solution at 3 g l<sup>-1</sup> to prevent fungal growth and desiccation. Plastic cups were capped with perforated lids to allow air flow and kept at room temperature until eclosion of adults the following season. At eclosion, species, number, and sex of emerged adults were recorded on a daily basis. All eclosed adults within 5 days of age were separated according to species and sex and placed in 3-l plastic cages provided with water and food (3:1 sugar:hydrolyzed protein) until sexual maturity (15–20 days) when they were used in experiments.

### Adult eclosion (allochronic isolation)

The number of days elapsed between pupation and adult eclosion was used to compare the duration of dormancy between species.

### Prezygotic isolation (observation cage, sexual behavior)

A cage of 1.08 m long, 1.08 m wide, and 1.78 m high was constructed using a 13 mm PVC pipe frame wrapped with white tergaline cloth. Within the cage, one 1.5-m-high potted oak tree (*Quercus* spp.) was placed at each corner, along with *J. pyriformis* branches pinned in circle on the cage walls and ceiling to simulate a tree canopy. Ten ripe *J. pyriformis* fruits were hung in circle from the cage ceiling using metal wire long enough to reach the potted tree foliage. *Juglans pyriformis* was chosen because fruit were readily available on the grounds of the botanical garden of the INECOL. Flies in the *R. suavis* species group appear to use various species of *Juglans* without distinction (Rull et al., 2013a). Fruit was evenly spaced and labeled with a 3-cm<sup>2</sup> green cardboard number to ease spatial location of activity. The observation cage was provided with water and food as described above.

At 09:00 hours 10 *R. zoqui* adult couples and 10 *R. ramosae* couples (20 couples in total) between 20 and 36 days of age, were released in the cage. Each individual fly was marked on the back of the thorax with a dot of water-based paint (Politec; Rodin, Mexico City, Mexico) using a single color or two-color combinations. Behavioral observations were performed by a single experienced observer from 10:00 to 17:00 hours with a combination of scanning and focal observation of key events (fruit and mate guarding, copulations, egg laying, etc.). Each fly cohort was observed for two consecutive days. A total of eight 2-day observations (replicates) were performed. Such observational methods have been used successfully to examine reproductive isolation among strains and species of fruit flies in the genera *Ceratitis*, *Bactrocera*, *Anastrepha*, and *Rhagoletis* (Cayol et al., 1999; McInnis et al., 1999; Rull et al., 2013b; Tadeo et al., 2013).

Male-female mating combination, hour of initiation and ending, and spatial location were recorded for every copulation. Fruit guarding was considered when a male remained motionless on a fruit for one or more minutes. For male-male encounters, the species, status (resident-intruder), location, and final outcome (considering the male remaining on the territory as the winner) of conflicts were recorded. Finally, in the case of females, fruit visits, egg laying events, and male rejections (brisk movements to prevent intromission after mounting) were recorded.

### Postzygotic isolation (hybrid mating fertility)

In order to compare fertility (egg hatch rates) among homo- and heterotypic *R. zoqui* × *R. ramosae* crosses, groups of five virgin females and five virgin males of between 20 and 38 days old were introduced in 3-l cages provided with water and food in all possible male-female mating combinations (r♂r♀, r♂z♀, z♂r♀, and z♂z♀). For

each one of the four mating combinations, couples were allowed to interact freely for 24 h and then provided with a 2.5-cm-diameter agar sphere for egg laying. The sphere was replaced in all cages on a daily basis for a 15-day period (15 spheres per cage across the observational period). All eggs in spheres were extracted and aligned over a dark piece of cloth placed over a moist piece of cotton within a Petri dish (Rull et al., 2010). Eggs were incubated at 24 °C for 6 days and observed under a dissecting microscope to calculate egg hatch percentage. The procedure was repeated 5× for each mating combination (five cages with five couples for every mating combination, a total of 20 cages).

#### Statistical analysis

Statistical comparisons made on the basis of frequencies were done using the total number of observations of a particular event per replicate. Time (days) from pupation to adult eclosion was compared between *R. zoqui* and *R. ramosae* by means of a Mann-Whitney U test. Frequency and duration (min) of copulations for each possible male-female *R. zoqui* × *R. ramosae* mating combination were compared by constructing a linear model with mating combination as a fixed factor and replicate (test date) as a random factor. Mating location (fruit or mesh) was also compared among male-female mating combinations with a mixed-effect linear model with replicate as a random factor. Frequency of fruit guarding according to species, joint fruit residency by males of same or different species, and the outcome of male-male conflicts (frequency of resident or intruder male remaining on fruit) were also analyzed by constructing linear models with replicate as a random factor. The frequency of male combats according to site (fruit vs. mesh) was compared by means of a t-test. The frequency of fruit visits, egg laying, and clutch size of females were compared between species using ranked data followed by a t-test. Egg hatch (%) for different male-female *R. zoqui* × *R. ramosae* mating combinations were compared by constructing a linear model with cage/cohort and sphere as random factors, mating combination as a fixed factor, and % egg hatch as the dependent variable. Analyses were performed with STATISTICA v.7 (TIBCO, Palo Alto, CA, USA) and SigmaPlot v.10.0 (Systat software, San José, CA, USA) software. Sexual isolation indices between *R. zoqui* and *R. ramosae* were calculated with JMATING software (Carvajal-Rodríguez & Rolan-Alvarez, 2006).

## Results

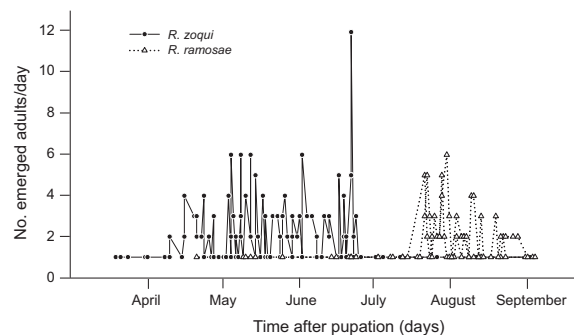
#### Adult eclosion

Out of 1 050 *R. zoqui* pupae recovered from fruit collections in Veracruz, 247 adults (133 males, 114 females) were

obtained, whereas of 725 pupae of *R. ramosae* from Guerrero, 130 adults (70 males, 60 females) eclosed. Seasonal adult eclosion patterns were clearly distinct for *R. zoqui* and *R. ramosae* ( $t = 10694.500$ , d.f. = 192,  $P < 0.001$ ; Figure 2). In the case of *R. zoqui*, the eclosion period lasted from 8 April to 25 June, whereas for *R. ramosae* it spanned from 17 July to 1 September. In sum, we detected a time gap between the end of *R. zoqui* and the beginning of *R. ramosae* adult eclosion of about 3 weeks.

#### Prezygotic isolation

The frequency of copulations differed among male-female *R. zoqui* × *R. ramosae* combinations ( $F_{3,21} = 3.99$ ,  $P = 0.021$ ), the effect of replicate was not significant ( $F_{7,21} = 0.62$ ,  $P = 0.072$ ). Homotypic combinations ( $zz = 4.375 \pm 1.281$ ,  $rr = 4.750 \pm 0.818$ ) and the  $z\sigma r\varphi$  hybrid cross ( $5.250 \pm 1.031$ ) occurred at similar frequencies; however, the  $r\sigma z\varphi$  hybrid cross was clearly less frequent ( $0.875 \pm 0.479$ ; Figure 3). A mixed-effect linear model did not reveal differences in copulation duration among pure and hybrid male-female mating combinations ( $F_{3,21} = 1.48$ ,  $P = 0.23$ ) and replicate had no effect on mating duration ( $F_{7,21} = 1.53$ ,  $P = 0.19$ ). For the  $z\sigma z\varphi$  combination, copulations lasted (mean ± SE =)  $14.51 \pm 0.92$  min,  $r\sigma r\varphi$  lasted  $15.89 \pm 1.28$  min,  $z\sigma r\varphi$   $17.26 \pm 1.24$  min, and  $r\sigma z\varphi$   $13.29 \pm 2.98$  min. With respect to mating frequencies according to location (fruit or cage mesh), a mixed-effect linear model revealed differences among *R. zoqui* × *R. ramosae* male-female mating combinations ( $F_{3,52} = 3.82$ ,  $P = 0.015$ ) and between mating sites ( $F_{1,52} = 22.47$ ,  $P < 0.001$ ). Replicate had no effect on mating location ( $F_{7,52} = 0.49$ ,  $P = 0.83$ ). The  $r\sigma z\varphi$  hybrid cross occurred at lower frequencies than the other mating combinations; 83.6% of copulations occurred on fruit and only 16.4% on the cage mesh. Overall, we



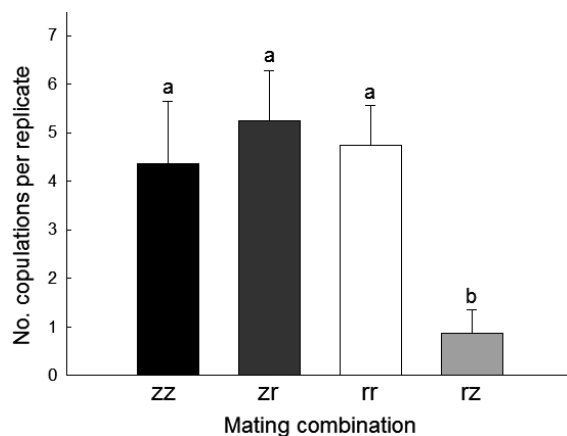
**Figure 2** Total number of eclosed adults of *Rhagoletis zoqui* (dots) from Veracruz collected from *Juglans pyriformis* and *R. ramosae* (triangles) collected in Guerrero from *J. major* over time.

observed significant levels of sexual isolation between *R. zoqui* and *R. ramosae* ( $I_{\text{PSI}} = 0.34$ ,  $P = 0.001$ ).

#### Male behavior

There were no differences in the frequency of fruit guarding between *R. zoqui* and *R. ramosae* male-male combinations ( $F_{1,7} = 0.65$ ,  $P = 0.45$ ) and replicate had no effect on fruit-guarding frequency ( $F_{7,7} = 2.46$ ,  $P = 0.12$ ). In general, males from both species exhibited similar fruit-guarding frequency (*R. zoqui*:  $24.13 \pm 4.09$ ; *R. ramosae*:  $20.88 \pm 3.35$ ). A mixed-effect linear model did not reveal differences in frequencies of joint fruit guarding among the various male-male combinations ( $F_{3,21} = 0.83$ ,  $P = 0.48$ ), and replicate had no effect either ( $F_{3,21} = 1.69$ ,  $P = 0.17$ ). On average (mean  $\pm$  SE), guarding frequency of *R. zoqui* males sharing fruit with other *R. zoqui* males was  $4.13 \pm 1.25$ , and sharing with *R. ramosae* males it was  $3.0 \pm 0.71$ , whereas the frequency of *R. ramosae* males encountering their conspecifics or *R. zoqui* on fruit was  $2.13 \pm 0.72$  and  $3.63 \pm 1.27$ , respectively.

The frequency of male contests differed between locations (fruit or mesh) ( $t = 3.663$ , d.f. = 16,  $P = 0.003$ ). On average, 87.3% of male-male encounters occurred on fruit, whereas the remaining 12.7% occurred on the cage walls or ceiling. There were no differences in frequency among male-male species combinations according to resident status ( $F_{3,21} = 0.92$ ,  $P = 0.44$ ). Replicate had an effect on contest frequency ( $F_{3,21} = 3.53$ ,  $P = 0.011$ ); overall activity was lower for replicates 1 and 6. Male contests for the zz combination occurred at an average frequency of  $16.50 \pm 4.71$ , contests for the zr resident-intruder combination at  $11.25 \pm 2.63$ , for the rr combination at  $11.75 \pm 2.58$ , and for the rz resident-intruder



**Figure 3** Mean (+ SE) frequency of copulation for various male-female pure and hybrid mating combinations between *Rhagoletis zoqui* (z) and *R. ramosae* (r). Means capped with different letters are significantly different (GLMM:  $P < 0.05$ ).

combination at  $14.75 \pm 2.87$ . Male status (resident/intruder) and species had no effect on the frequency of male-male contest victories (a particular male remaining on fruit after a contest) ( $F_{3,21} = 1.00$ ,  $P = 0.43$ ). Replicate had an effect on frequency of male victory ( $F_{3,21} = 7.39$ ,  $P < 0.001$ ), but not the interaction between male status and replicate ( $F_{42,40} = 1.36$ ,  $P = 0.16$ ). A total of 17.4% of male-male contest victories was for resident *R. zoqui*, 14.7% for *R. zoqui* intruders, 18.2% for resident *R. ramosae*, 10.7% for intruder *R. ramosae*, 13.1% for both *R. zoqui* male types, 7.2% for both *R. ramosae* male types, and 18.7% for two males of different species (*R. zoqui* and *R. ramosae*). There were evident behavioral differences between species during male contests: *R. zoqui* typically lifted their wings showing their patterns, whereas *R. ramosae* males were static in an apparent state of alert (Figure 4). During contests, *R. ramosae* males lifted their wings in a  $45^\circ$  angle with respect to the thorax whereas *R. zoqui* males kept their wings somewhat folded (Figure 5).

#### Female behavior

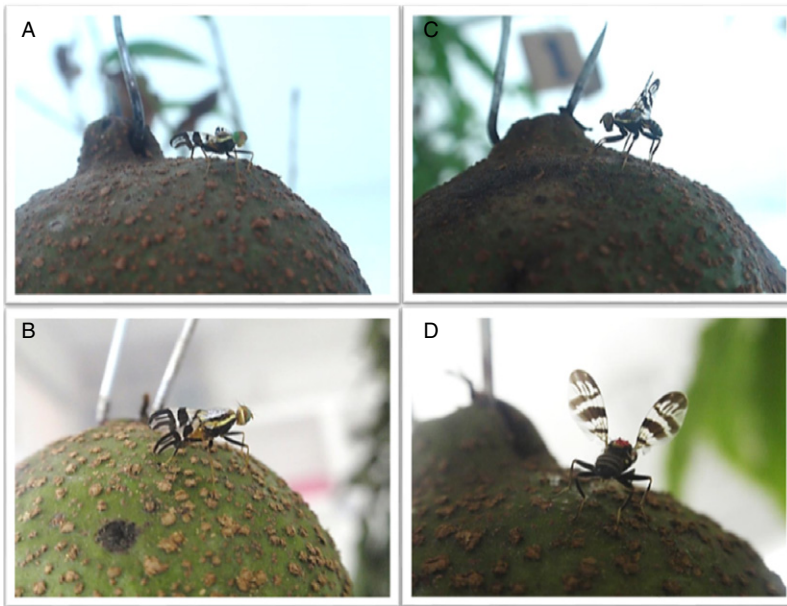
The frequency of fruit visits did not differ between species ( $t = 0.403$ , d.f. = 92,  $P = 0.69$ ) but the frequency of egg-laying was different ( $t = 3.149$ , d.f. = 92,  $P = 0.002$ ). *Rhagoletis zoqui* females performed a total of 381 visits and 32 egg depositions, with (mean  $\pm$  SD =)  $5 \pm 9.94$  and  $0 \pm 1.28$  egg laying bouts per female per replicate, whereas *R. ramosae* females visited fruit 456 $\times$  and laid eggs on 116 occasions, corresponding to  $7 \pm 84$  and  $1 \pm 2.84$  egg laying bouts per female per replicate.

#### Postzygotic isolation

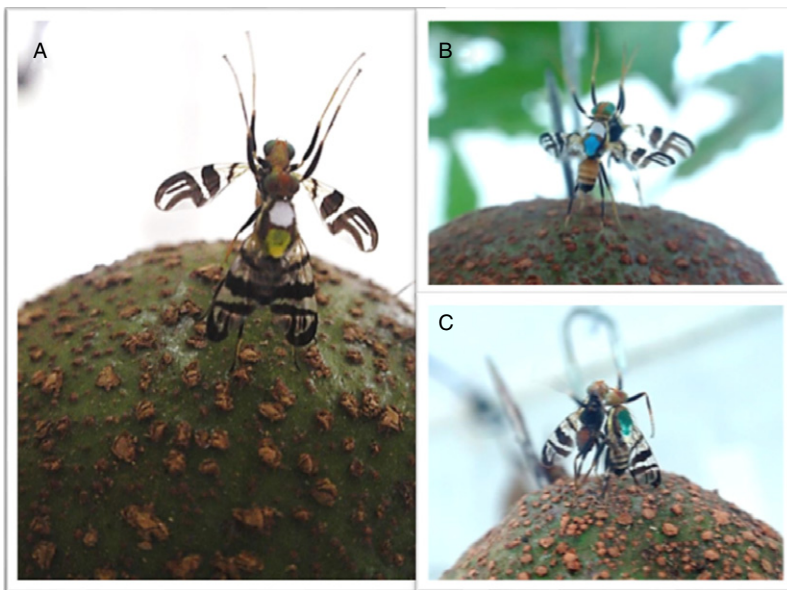
The number of eggs laid per female per egg-laying bout differed between species ( $t = -4.060$ , d.f. = 267,  $P < 0.001$ ). Female *R. zoqui* laid 1–11 eggs – on average (mean  $\pm$  SD =)  $5.41 \pm 2.31$  eggs per bout – whereas *R. ramosae* females laid 1–22 eggs, on average  $10.46 \pm 3.78$ . In total *R. zoqui* laid 617 eggs and *R. ramosae* 1 611 eggs, representing 27.7 and 70.3% across the entire study, respectively. Egg hatch differed among male-female *R. zoqui*  $\times$  *R. ramosae* mating combinations ( $r\sigma r\sigma$ ,  $r\sigma z\sigma$ ,  $z\sigma r\sigma$ , and  $z\sigma z\sigma$ ) ( $F_{3,94} = 6.81$ ,  $P = 0.003$ ) – cage/cohort ( $F_{4,94} = 1.88$ ,  $P = 0.12$ ) and sphere ( $F_{14,94} = 6.81$ ,  $P = 0.09$ ) had no effect. Eggs laid by females from the hybrid cross  $r\sigma z\sigma$  hatched the best; significantly fewer eggs laid by females from the hybrid cross  $z\sigma r\sigma$  and  $z\sigma z\sigma$  hatched (Figure 6).

#### Discussion

In congruence with phylogenetic studies and previous reproductive compatibility tests among walnut-infesting



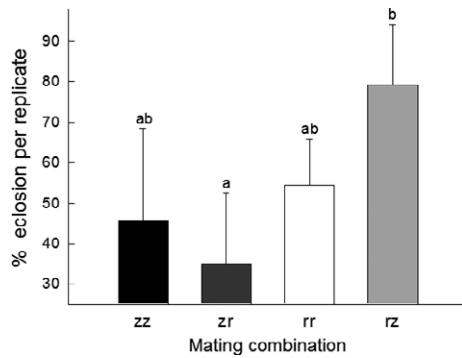
**Figure 4** Typical fruit guarding postures by (A, B) *Rhagoletis ramosae* and (C, D) *R. zoqui* males.



**Figure 5** Typical wing postures of (A) a *Rhagoletis zoqui* male (folded wings) vs. a *R. ramosae* male (extended wings), (B) two *R. ramosae* males (both contestants with extended wings), and (C) two *R. zoqui* males (both contestants with folded wings) during male-male contests on host fruit.

flies in the ‘*R. completa*, *R. zoqui*, *R. ramosae*’ clade, *R. ramosae* and *R. zoqui* were found to be capable of hybridizing. These species, however, displayed some degree of precopulatory mating (sexual) isolation, slightly greater than that found between *R. completa* and *R. zoqui* (Tadeo et al., 2013) and smaller than that found between *R. completa* and *R. ramosae* [*R. completa* vs. *R. zoqui*:  $I_{PSI} = 0$  (Tadeo et al., 2013); *R. zoqui* vs. *R. ramosae*:  $I_{PSI} = 0.34$ ; *R. completa* vs. *R. ramosae*:  $I_{PSI} = 0.53$  (E Tadeo, M Aluja & J Rull, unpubl.). In sum we found

evidence of host-plant-phenology-driven local-adaptation differences between *R. zoqui* and *R. ramosae* that result in partial allochronic isolation. We also found some degree of prezygotic mating isolation with *R. zoqui* females being more reluctant to mate with *R. ramosae* males than with males of their own species. The bulk of reproductive activities (male guarding, male contests, mating, egg laying) took place on the host fruit, with no evidence of alternative mating locations being used, as found for *R. completa* (Tadeo et al., 2013, E Tadeo, M Aluja & J Rull, unpubl.).



**Figure 6** Mean ( $\pm$  SE) egg hatch (%) for pure (zz, rr) and hybrid (zr, rz) crosses between adult *Rhagoletis zoqui* and *R. ramosae*. Means capped with different letters are significantly different (GLMM:  $P < 0.05$ ).

Distinctive behavioral differences were observed between males during guarding, contests, and approach to conspecifics on fruit. There was also some asymmetric postzygotic isolation, with lower egg hatch in the hybrid combination of *R. zoqui* males and *R. ramosae* females than in the other mating combinations.

Our results unveiled the existence of pre- and postzygotic barriers to gene flow between two endemic parapatric species of walnut-infesting *Rhagoletis* in Mexico that have maintained their integrity (clear morphological differences) despite contact. The first barrier is constituted by clearly defined adult eclosion periods tightly linked to differences in the fruiting phenology of their respective walnut hosts within their distributional ranges (*R. ramosae* [*J. major* and *R. zoqui*] [*J. pyriformis*]) (see also Rull et al., 2013a). As a result there is a 3-week temporal gap when adults of both species do not overlap from the end of the *R. zoqui* adult eclosion period to the beginning of the *R. ramosae* eclosion period. It has been proven for other species of *Rhagoletis* that diapause duration is genetically determined, highly variable, and that it responds to selection imposed by host plant fruiting phenology (Feder et al., 1997; Prokopy & Papaj, 2000). Synchronization between host plant phenology and overwintering is a key feature affecting survival and offspring fitness among specialized phytophagous insects with affinity for temperate climates (Feder et al., 1997; Prokopy & Papaj, 2000; van Asch & Visser, 2007). Host plant phenology can have evolutionary consequences. Feder et al. (1997, 2003) established that a shift in the fruiting period of host plants of *R. pomonella* (2–3 weeks), in addition to allelic changes in host-associated populations, played an important role in host race formation leading to speciation. Temporal isolation between host races and closely related species has been documented for several species of phytophagous insects

(Drès & Mallet, 2002; Matsubayashi et al., 2010). Walnut-infesting *Rhagoletis* can survive as adults in the laboratory for 40–70 days (Guillen et al., 2011). Although allochronic isolation is not complete it may contribute to reducing geneflow between *R. ramosae* and *R. zoqui*.

We found evidence of asymmetric sexual isolation (sensu Kaneshiro, 1983) during behavioral observations with *R. zoqui* females preferring to mate with conspecifics over *R. ramosae* males. In contrast, *R. ramosae* females mated with males of both species. Asymmetric sexual isolation has been documented between other closely related species pairs in the *suavis* group (*R. zoqui* and *R. completa*) (Tadeo et al., 2013) to a lesser degree than what we observed between *R. zoqui* and *R. ramosae*. Sexual selection has been claimed to be one of the most important forces triggering divergence and speciation between populations with allopatric history (Ritchie, 2007; Jennings et al., 2011). Additionally, prezygotic barriers to gene flow may have a stronger effect than postzygotic barriers in generating reproductive isolation (Coyne & Orr, 2004; Cuevas, 2012), as they prevent the waste of costly gametes in production of hybrids with lower fitness (Kaneshiro, 1976).

Although male behavior of both *R. zoqui* and *R. ramosae* adjusted to general patterns summarized by Prokopy & Papaj (2000) for several species in the genus *Rhagoletis* (males defending fruit from rivals to force copulations on females seeking to oviposit), some distinctive behavioral displays and tactics were observed. An example of the latter may be the degree of aggressiveness or tolerance to the presence of other males displayed by *R. zoqui* and *R. ramosae* during fruit guarding, which often resulted in two males occupying the same territory (fruit). Tadeo et al. (2013) had observed this behavioral pattern for some *R. zoqui* males which could constitute an alternative mating tactic used to remain on fruit by avoiding contests with more aggressive or otherwise superior males and thus maintain access to females visiting fruit for egg-laying. Our study constitutes one of the few behavioral descriptions for *R. ramosae* (besides E Tadeo, M Aluja & J Rull, unpubl.), a species for which there are only published records of taxonomy, parasitism, distribution, and phenology (Hernández-Ortiz, 1985; Ovruski et al., 2007; Rull et al., 2013a). Other interesting details of mating behavior observed here were the differences in male displays during fruit guarding when facing an intruder (male or female, conspecific or not). Male *R. zoqui* almost invariably held their wings lifted perpendicular to the thorax in a display termed ‘lofting’ (Headrick & Goeden, 1994), also exhibited by *R. completa* (Tadeo et al., 2013), but not by *R. ramosae*. *Rhagoletis ramosae* males remained motionless in an alert posture with their wings folded. Another relevant observed

trait is the way *R. ramosae* males open their wings during male-male combat, a behavior also regularly displayed by *R. boycei*, a member of the *suavis* group (Rull et al., 2013a). Flies in the genus *Rhagoletis* exhibit a resource defense mating system where males guard and defend fruit where they force egg-laying females to copulate (Prokopy & Papaj, 2000). Accordingly, the behavioral differences we observed between males should not produce prezygotic isolation because they are not an element of courtship, unless females are able to detect males on guarded fruit before landing where the guarding posture of a heterospecific male could have a deterring effect. This hypothesis, however, requires appropriate testing.

We observed significant differences in hatching percentage for eggs laid by the *R. zoqui* male  $\times$  *R. ramosae* female hybrid mating combination (zr), yet these were only numerically inferior to those recorded from eggs recovered from both pure crosses (zz and rr) and significantly inferior to egg hatch of the rz hybrid cross. In contrast, Rull et al. (2012) found no differences in hatching among eggs laid by pure and hybrid *R. zoqui*  $\times$  *R. completa* crosses, two species that hybridize in nature. In the case of *R. zoqui*  $\times$  *R. ramosae* hybrid combinations, the observed pattern (asymmetric cytoplasmic incompatibility) is a common outcome in crosses among strains and species infected and uninfected with *Wolbachia* or carrying different strains of the bacterium (Werren et al., 2008), and could be indicative of *R. zoqui* infection. However, it is important to note that the hybrid cross yielding the highest levels of egg hatch was the one that was the least likely to occur during prezygotic isolation tests, a result contrary to findings for uninfected and infected sympatric pairs of *Drosophila* species, where uninfected females were selected to discriminate against infected males in areas of sympatry (Jaenike et al., 2006). Although *Wolbachia* plays a fundamental role in the reproduction of *Rhagoletis* flies in the *pomonella* and *cingulata* species group, in the *suavis* group it has been concluded that *R. completa* is *Wolbachia* free (Drosopoulou et al., 2010) or harbors low titers of *Wolbachia* that appear to have no phenotypic effects (Schuler et al., 2012). *Wolbachia* screening of several species of the *R. suavis* group (including *R. zoqui*) has revealed that all species of walnut-infesting *Rhagoletis* examined carry very low titers of *Wolbachia* that very likely do not cause cytoplasmic incompatibility (Hannes Schuler, pers. comm.).

*Rhagoletis zoqui* and *R. ramosae* displayed a higher degree of reproductive incompatibility than the one observed between *R. zoqui* and *R. completa*. We believe that biological differences between *R. zoqui* and *R. ramosae* could have arisen during geographic isolation as a result, in part, to adaptation to the fruiting

phenology of their respective host plants, distinct environmental conditions, but also as a result of inter-specific selection on male displays. Prezygotic isolation could have been reinforced during periods of secondary contact producing patterns documented here by altering female preferences in some manner. According to Coyne & Orr (1989) both pre- and postzygotic isolation in *Drosophila* increase with divergence time between taxa. If such a pattern holds true for flies in the genus *Rhagoletis*, our results and those of Rull et al. (2012) and Tadeo et al. (2013) would support a closer relationship between *R. zoqui* and *R. completa* than between *R. zoqui* and *R. ramosae*. It would be interesting to complete studies on reproductive compatibility among Mexican species in the *R. suavis* group by examining interactions between *R. ramosae* and *R. completa* and *R. ramosae* and *R. boycei*. According to a recent phylogeny of the *suavis* group (Glover et al., 2018), and the hypothesis put forth by Coyne & Orr (1997), reproductive isolation between *R. boycei* and members of *R. ramosae* and *R. completa* and *R. ramosae* clade should be stronger than those reported here. Similarly, across the entire range of the *suavis* group, *R. suavis* and *R. juglandis* should be the most isolated species. Altogether, complete examination of reproductive isolation within walnut-infesting *Rhagoletis* could yield a comprehensive picture of speciation for a taxonomic group other than *Drosophila* on which most knowledge on the time course of speciation is currently based (Coyne & Orr, 1997).

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