Host plant stimuli effects on survival and duration of dormancy of *Rhagoletis zoqui*, *Rhagoletis completa* (Diptera: Tephritidae) and associated parasitoids

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Abstract

Tephritid fruit flies in the genus *Rhagoletis* bridge between predictable periods of fruit availability by becoming dormant. To cope with acyclic unpredictable events (e.g., frost, mast seeding, etc), a proportion of the population can undergo prolonged dormancy. In the case of walnut infesting *Rhagoletis*, host plant-derived cues such as juglone soil concentration vary seasonally in predictable patterns. Here, we examined the effects of host plant parts and derived compounds on emergence rates and dormancy duration of *Rhagoletis completa* (Cresson), *Rhagoletis zoqui* (Bush) (Diptera: Tephritidae), and associated parasitoids. Pupae of both species were exposed to walnut leaves, fruit, or fruit and leaves and compared to a control. In a second experiment, *R. zoqui* were exposed to 10 mg l^{-1} of juglone applied to pupation medium during four consecutive 4-week time periods under variable combinations of temperature and frequency of exposure. Overall, the presence of fruit resulted in greater overwintering survival of *R. completa* but had no effect on the duration of dormancy of either fly species. Application of juglone over two consecutive periods produced greater mortality of *R. zoqui* than the control. Three parasitoid species emerged from *R. completa* and one from *R. zoqui*. Duration of dormancy for parasitoids was longer than that of fly hosts. Regardless of treatment, 13.3–18.4% of *R. completa* pupae and 1.3–2.8% *R. zoqui* engaged in prolonged (>year) dormancy. Our results indicate that host plant derived cues have little or no effect on survival and duration of dormancy of walnut infesting *Rhagoletis*, and at the tested concentration juglone is toxic to *R. zoqui* pupae. Testing the effect of juglone at lower concentrations is necessary to rule out its role as an environmental cue for regulation of dormancy. So far, host plant fruiting phenology appears to play a greater role than host plant derived cues in selecting for fly eclosion times.

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Introduction

Most tephritid fruit flies within the genus *Rhagoletis* are univoltine, specialized stenophagous frugivores that exploit groups of plants with discrete yearly phenologies (Bush, 1966). Adults must emerge in synchrony with ripening fruit on which they meet, mate, and deposit eggs to give rise to a new generation (Prokopy and Papaj, 2000). Flies synchronize emergence with suitable host fruit through dormancy (Bush, 1966; Boller and Prokopy, 1976; Prokopy and Papaj, 2000). This life-history strategy enables flies to exploit relatively predictable and seasonal host plants (Bush, 1966). To cope with inter annual variability, (e.g., early frost events during flowering or fruit set, or insufficient, or extended chill periods), a proportion of the population can engage in prolonged dormancy of variable duration (2, 3, 4, or 5 years) (Boyce, 1931; Dean, 1973), presumably as a bet hedging strategy (Moraiti *et al.*, 2014; Rull *et al.*, 2018). Dormancy regulation (induction, duration and termination) and overwintering survival in *Rhagoletis* have been found to be influenced by environmental factors such as winter length, temperature, relative humidity, soil moisture, photoperiod, and light intensity (Neilson, 1962, 1964; Prokopy, 1968; Baker and Miller, 1978; Feder *et al.*, 1997; Filchak *et al.*, 2001; Yee, 2013).

Host plant phenology has also been found to affect the duration of dormancy among host races (morphologically similar species exploiting different closely related host plants, e.g., apple/hawthorn) and sister species (morphologically similar species exploiting different plant families, e.g., Rosaceae/Ericaceae) of *Rhagoletis* (Berlocher, 2000) resulting in allochronic isolation among diverging taxa (Smith, 1988; Feder and Filchak, 1999). Quantitative and qualitative changes in the condition of host plants can provide signals for dormancy regulation among some phytophagous insects (Denlinger, 1986). For example, Hodek *et al.* (1981) found that the presence of pollen induces adult diapause in the Mexican bean beetle

Acanthascelides obtectus, while senescing potato leaves have been found to induce diapause in the Colorado potato beetle *Leptinotarsa decemlineata* (cited in Gill *et al.*, 2017). Nevertheless, the influence of host plant derived seasonal chemical cues (detectable plant derived compounds that appear or vary over the season in a predictable way) on diapause of *Rhagoletis* spp., or any other species of Tephritidae, has thus far not been explored.

Within the genus *Rhagoletis*, six described species in the *Rhagoletis suavis* species group exploit plants in the genus *Juglans* (Bush, 1966; Rull *et al.*, 2013). Host fruit size and the presence of conspecific larvae within it have been found to influence the reproductive behaviour of some members of the *R. suavis* group (Papaj, 2005). *Rhagoletis juglandis* (Cresson) females exposed to host fruit (colour and shape stimuli) exhibit greater ovarian development than unexposed flies (Alonso-Pimentel *et al.*, 1998). In walnut infesting *Rhagoletis* species, females do not avoid laying eggs in infested fruit, where usually several larvae develop without interference (Nufio *et al.*, 2000). Feeding of tephritid larvae within fruit enhances the breakdown of phenolic compounds and tannins in comparison to non-infested walnuts (Oroño *et al.*, 2019), which suggests that this behavioural trait among walnut infesting *Rhagoletis* may be an adaptation to the secondary plant compounds within ripening walnut fruit.

Juglone is a phenolic compound present in the walnut husk and leaves, but also released by the plant root system (Cosmulescu *et al.*, 2011). Because juglone is poorly soluble in water, it does not move far in the soil (Dana and Lerner, 2001). However, juglone is more persistent in poorly drained soils than in well drained soils (Willis, 2000). Juglone is both microbially and abiotically degraded (Von Kiparski et al., 2007) and therefore its concentration in the plant and soil is both dynamic and seasonal (Coder, 1983). Juglone concentration in the soil is highest under the tree canopy and decreases as much as 80% when measured 4.25 m away from the tree (Jose and Gillespie, 1998). Juglone concentration under the tree canopy is probably influenced by the combined release of leaves, fruit and the root system, which all contain and release this compound (Cosmulescu *et al.*, 2011). Although most detailed studies on juglone soil dynamics have been made with *Juglans nigra* and *Juglans regia*, there is evidence for non-commercial species of walnut with allelopathic effects (Willis, 2000). Juglone concentration in the soil peaks during fall through release by decaying fruits and leaves, drops in winter, rises in spring, and decreases during the summer (de Scisciolo *et al.*, 1990). These seasonal patterns are related to fruit and leaf drop but also by release by the root system. Additionally, plants in the genus *Juglans* are known to engage in mast seeding (the synchronous intermittent production of large seed crops) (Stapanian and Smith, 1978; Kelly and Sork, 2002), which results in inter annual variability in fruit availability, and probably increases in juglone soil concentration. Such trait (masting), could select for prolonged dormancy among walnut infesting *Rhagoletis*.

Rhagoletis completa (Cresson) and *Rhagoletis zoqui* (Bush) are important pests of walnuts in Mexico and other parts of the world (Yee *et al.*, 2014). *R. completa* in Mexico is typically found infesting *Juglans hirsuta*, *J. mollis*, and *J. regia* (Rull *et al.*, 2013), while *R. zoqui* has been recovered from *J. mollis*, *J. pyriformis*, and *J. regia* (Rull *et al.*, 2013). For both species, most of the individuals in the population are univoltine and under natural uncontrolled environmental conditions roughly take between 250 and 300 days from pupation to adult eclosion independently of walnut host species origin (Rull *et al.*, 2013). Both species become dormant as pupae to synchronize adult emergence with seasonal host fruit availability (Rull *et al.*, 2016, 2019a). Two parasitoid species have been found in exclusive association with walnut infesting *Rhagoletis*, *Aganaspis alujai* and *Diachasmimorpha juglandis* (Rull *et al.*, 2013), both parasitoids also become dormant and emerge as adults later than their fly hosts (Rull *et al.*, 2019a). Considering the tight relationship between the phenology of *Juglans* fruits and adult emergence of walnut infesting *Rhagoletis,* here we tested the hypothesis that host plant chemicals influence adult emergence rates and emergence time of both species and their parasitoids. Given that larvae feed on the husks of walnuts, which are known to have high concentrations of this compound (Cosmulescu *et al.*, 2011), we exposed *R. zoqui* and *R. completa* pupae in vermiculite to a warm (24°C) followed by a cold (5°C) and two consecutive warm 4-week periods. Temperature regimes were established to mimic seasonal variation in temperature (fall, winter, spring, summer) and included a chill period necessary to break diapause. Such pupae were exposed to combinations with and without walnut fruit and/or leaves. Additionally, a second experiment was set up to directly expose *R. zoqui* pupae to different combinations with and without a 3 μg of juglone per g of vermiculite.

Materials and methods

Biological material

Rhagoletis zoqui was obtained from fruit of *Juglans pyriformis* Liebmann collected on the old Xalapa-Coatepec road (19° 29′8.15″N, 96°56′42.01″W; 1334 m a.s.l.) under the canopy of several trees during the 2nd and 3rd week of September 2017 and 2018. *Rhagoletis completa* was obtained from a single collection of *Juglans hirsuta* Manning in the locality of San Juan Bautista, Nuevo León (25°23′36.6″ N, 100°18′05.1″ W; 1385 m a. s. l.) during the 1st week of September 2017. No *R. completa* could be collected in 2018, the collection site is more than 1000 km away from the Xalapa laboratory, and it was difficult to predict the timing of fruit drop. Collected fruits were taken to the laboratory at the Instituto de Ecología AC and handled as described by Rull *et al.* (2006) to recover pupae. Pupae were removed from trays every 3–4 days and placed in groups of 50 in transparent plastic 200 ml cups containing 5 g of vermiculite previously moistened with a sodium benzoate solution (3 g/l) applied with a hand sprayer to hinder fungal growth. All pupae were of similar age (1–4 days after pupation) when assigned to treatments. Cups were covered with an aerated mesh lid and taken to a laboratory under controlled environmental conditions at a 24 ± 1°C, a 65 ± 5% RH and a 12/12 light/dark (L/D) photoperiod for 7 days.

Host plant parts

For each *Rhagoletis* species, a total of 36 cups, with 50 pupae each (1800 total pupae, 450 pupae per treatment, subdivided in nine plastic cups with 50 pupae each) were gathered and transferred to 0.7 litre plastic cups with 20 g of vermiculite according to four different treatments with nine replicates each. Treatments were: (i) vermiculite alone, (ii) vermiculite with the addition of five leaves of *J. pyriformis* (total weight between 1.6 and 2 g), (iii) vermiculite with the addition of one walnut fruit (fresh fruit weight between 70 and 90 g) and (iv) vermiculite with the addition of five leaves and one fruit (similar weight range). Leaves and fruits were collected form *J. pyriformis* trees at the same time. Fruits were covered with nylon gauzes in order to

avoid potential contamination of the experimental sample with larvae stemming from an infested fruit. All cups remained under a pre-winter period of 4 weeks. Thereafter, *R. zoqui* or *R. completa* pupae were transferred to a conventional refrigerator for a 'winter' period of 4 weeks. After the winter period, cups were returned to the lab, and kept under similar conditions as the pre-winter period to record emergence of adults after dormancy. Temperatures during periods 1, 3 and 4 were set in the lab at 24 ± 1°C; 65% RH, 12/12 L/D photoperiod, and for period 2, cups were transferred to a conventional refrigerator (Whirlpool Multi-flow WT1818A) at 5 ± 1°C; 65% RH, 0/24 L/D photoperiod.

Exposure to juglone

The juglone exposure experiment was only carried out with *R. zoqui* pupae obtained in 2018 as described above. No *R. completa* could be collected that season, due to the fact that the collection site is located ca. 1200 km away from Xalapa. A total of 84 cups, with 30 pupae each, were prepared and distributed according to six different treatments and 14 replicates per treatment (a total of 2520 pupae, 420 pupae per treatment). Following the 1st week after collection, all cups were exposed for a complete set of four 4-week periods under warm (24°C – period 1), followed by a cold (5°C - period 2), and two consecutive warm periods (3 and 4). The six treatments were: (i) vermiculite without juglone for the entire period (water control), (ii) vermiculite treated with juglone for the whole period, (iii) vermiculite treated with juglone during periods 1 and 3, (iv) vermiculite treated with juglone during periods 1 and 2, (v) vermiculite treated with juglone during periods 3 and 4, and (vi) vermiculite treated with juglone during periods 2 and 4 (table 1).

Cups were moistened once a week with 1.5 ml of a sodium benzoate solution (3 g/l) + 2% ethanol (control) or with a solution of sodium benzoate (3 g/l) that contained 10 mg/l of juglone (Sigma Aldrich, México) diluted in 2% ethanol. Ethanol occurs naturally in ripe and decaying fruit at concentrations ranging from 0.03 to 8.1% (Dudley, 2004). Tephritid larvae and other herbivores are therefore normally exposed to this compound without suffering mortality. However, because ethanol was used in all treatments, including the water control, differences in survival and eclosion time of exposed pupae could be attributed to different treatments. In the case of juglone treated cups, each application corresponds to approximately 3 µg of juglone per g of vermiculite, a concentration previously reported in soils under black walnut (*Juglans nigra* L.) trees (de Scisciolo *et al.*, 1990). After the final 4-week period 4, all cups were treated with a control sodium benzoate solution (3 g/l) on a weekly basis until the end of the experiment. Temperatures during periods 1,3 and 4 were set in the lab at 24 ± 1°C; 65% RH, 12/ 12 L/D photoperiod, and for period 2, cups were transferred to a conventional refrigerator at 5 ± 1°C; 65% RH, 0/24 L/D photoperiod.

Data recording

For both experiments, emergence date and sex of flies and parasitoids were recorded twice a week (Monday and Friday) until the experiment was terminated 1 year after pupae were collected. The duration of the pre-winter period (ca. 40 days from fruit collection) was established to record proportions of non-dormant flies (Rull *et al.*, 2016). The emergence time was estimated as the length of time (days) from the end of the artificial winter (period 2) to adult emergence. At the end of the experiment, 1 year after pupal collection, pupae for all treatments and replicates

Table 1. Four-week time periods under juglone treatment [water (white)/ juglone (light grey)] applied to 50-pupae lots of *Rhagoletis zoqui*

Treatment	Warm (24 ± 1°C) 4 weeks	Cold (5 ± 1°C) 4 weeks	Warm (24 ± 1°C) 4 weeks	Warm (24 ± 1°C) 4 weeks
T1	Water	Water	Water	Water
T2	*Juglone*	*Juglone*	*Juglone*	*Juglone*
T3	*Juglone*	Water	*Juglone*	Water
T4	*Juglone*	*Juglone*	Water	Water
T5	Water	Water	*Juglone*	*Juglone*
T6	Water	*Juglone*	Water	*Juglone*

were inspected under a dissecting microscope to establish the proportion of live pupae and the proportion of dead pupae. Time to emergence for flies and parasitoids was corrected for winter length (subtracted). Parasitoids were identified using morphological traits by one of us (LG). The rate of parasitism (percent) was calculated two ways in reference to both the total number of pupae and by dividing the number of emerged parasitoids over the sum of emerged parasitoids and emerged flies.

Statistical analyses

Cumulative percentages of adult emergence for *R. completa* and *R. zoqui* were compared among treatments and sex using a generalized linear model (GLM) with normal distribution (*R. completa*: Kolmogorov–Smirnov $d = 0.089$, $P = 0.729$; *R. zoqui*: Kolmogorov-Smirnov $d = 0.062$, $P = 0.988$) followed by Tukey's honestly significant difference (HSD) tests. The emergence time of *Rhagoletis* pupae was compared among treatments and sex by means of GLMs with quasi-Poisson distribution for over dispersion, followed by Bonferroni mean comparisons. Percentages of live uneclosed pupae (failing to yield adult flies) at the end of experiment were compared among treatments, for each species, through Chi-squared tests with binomial error distribution.

Cumulative percent overall adult parasitoid emergence was compared among treatments by means of a GLM with normal distribution (Kolmogorov-Smirnov: $d = 0.101$, $P = 0.854$) followed by Tukey's HSD mean comparisons. Mean length of post-winter adult dormancy was compared among treatments for each separate parasitoid species with an analysis of variance (ANOVA) in the case of parasitoids in the genus *Diachasmimorpha* and a non-parametric Kruskal Wallis test for *Aganaspis alujai* (Wharton & Ovruski). Comparison of mean length of dormancy between both parasitoid genera was subjected to a Mann–Whitney *U* test. Cumulative percent of adult emergence and emergence time of *R. zoqui* pupae exposed to juglone was compared among treatments with a two-way ANOVA with treatment and sex as factors. Statistical analyses were all performed using the R-based program Jamovi v.0.9.1.12 (Jamovi, 2019) or with the Generalized Linear Interactive Modelling (GLIM) program (Numerical Algorithms Group, 1993).

Results
Host plant parts
Emergence rates of R. completa *and* R. zoqui *pupae*
No *R. completa*, *R. zoqui*, or parasitoid adults emerged from cups without becoming dormant during the 40-day pre-winter period in the host plant cue experiment ($n = 1800$ total pupae).
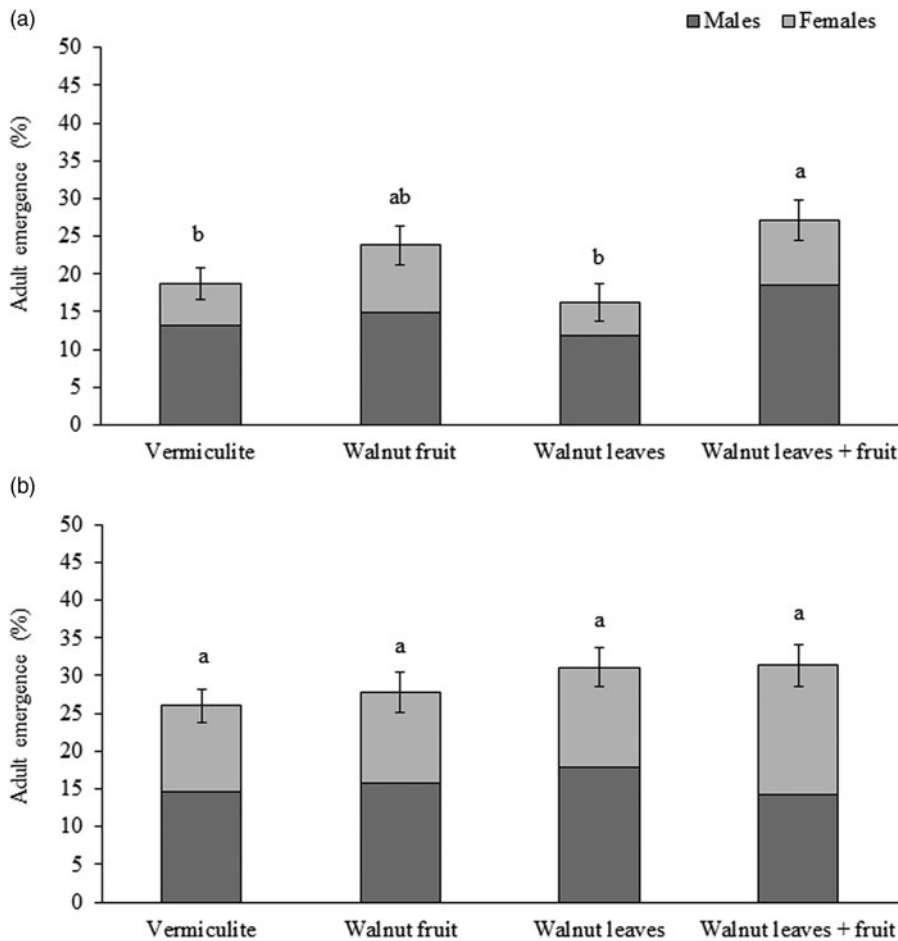
Figure 1. Mean (±S.E.) percent emerged fly adults (males in dark grey bars followed by females in clear grey bars) for (a) *Rhagoletis completa* (N = 262 males, 124 females) and (b) *Rhagoletis zoqui* (N = 281 males, 242 females) in reference to pupae exposed to vermiculite alone or vermiculite with a fruit, leaves or leaves plus a fruit. Different letters above bars indicate significant differences (P < 0.05), Tukey HSD.

For *R. completa*, after the pre-winter period, a total of 385 adult flies emerged from all pupae reared in the experiment (36 cups, 50 pupae each one, overall 21.4% adult emergence), with 262 males (68%) and 124 females (32%). For males, emergence rates ranged from 11.8 ± 1.4% (mean ± SE) (walnut leaves) to 18.4 ± 2.5% (mean ± SE) (walnut leaves + fruit). For females, emergence rates ranged from 4.4 ± 1.1% (mean ± SE) (walnut leaves) to 8.9 ± 1.6% (mean ± SE) (walnut fruit).

Significant differences were observed in the percentages of *R. completa* emergence among treatments ($F = 6.301$; $df = 3,64$; $P < 0.001$) (fig. 1a). Pupae exposed to vermiculite containing walnut leaves and fruit emerged in the highest percentages, followed by pupae in vermiculite with walnut fruit with intermediate percentages and vermiculite alone or vermiculite containing walnut leaves with the lowest percentages. There was also a statistically significant effect of sex on adult percentage emergence ($F = 13.147$; $df = 1,64$; $P < 0.001$), but no significant interaction between sex and treatment ($F = 0.137$; $df = 3,64$; $P = 0.937$).

For *R. zoqui*, after the pre-winter period, a total of 523 adult flies emerged from all pupae reared in the experiment (36 cups, 50 pupae each one, representing an overall 29.1% adult emergence), with 281 males (54%) and 242 females (46%). For males, emergence rates ranged from 14.2 ± 1.6% (mean ± SE) (walnut leaves + fruit) to 17.8 ± 2.8% (mean ± SE) (walnut leaves). For females, emergence rates ranged from 12.0 ± 1.5% (mean ± SE) (walnut fruit) to 17.8 ± 2.8% (mean ± SE) (walnut leaves + fruit). No significant differences were observed in percentage *R. zoqui* adult emergence for pupae exposed to a control treatment or different host stimuli ($F = 0.988$; $df = 3,64$; $P = 0.404$) (fig. 1b). There was no statistically significant effect of sex ($F = 2.594$; $df = 1,64$; $P = 0.112$) or for the interaction between sex and treatment ($F = 0.1502$; $df = 3,64$; $P = 0.222$).

Emergence time of R. completa *and* R. zoqui *pupae*
For *R. completa*, the mean emergence time for males ranged from 136 to 148 days, and for females it ranged from 134 to 164 days. The mean emergence time for *R. completa* adults was not statistically different among treatments ($\chi^2 = 4.75$; $df = 3$; $P = 0.191$). No statistical difference was observed between sexes ($\chi^2 = 0.071$; $df = 1$; $P = 0.790$) or the interaction between sex and treatment ($\chi^2 = 1.2$; $df = 3$; $P = 0.240$) (fig. 2a).

For *R. zoqui*, the mean emergence time for males ranged from 119 to 126 days, and for females it ranged from 105 to 114 days. The mean emergence time for *R. zoqui* adults was not statistically different among pupae subjected to a control treatment or different host stimuli ($\chi^2 = 2.84$; $df = 3$; $P = 0.418$) (fig. 2b). However, the mean emergence time for males ∼122 days was statistically different than for females ∼111 days ($\chi^2 = 10.54$; $df = 1$; $P < 0.001$). No significant interaction between sex and treatment was observed ($\chi^2 = 1.17$; $df = 3$; $P = 0.167$) (fig. 2b).

The percentages of *R. completa*, pupae that remained alive at the end of experiment ranged from 13.3% to 18.4% among treatments. However, the variation among treatments was not statistically significant ($\chi^2 = 5.22$; $df = 3$; $P = 0.16$) (fig. 3a).

The mean percentage of *R. zoqui* pupae that remained alive at the end of experiment among treatment of the effects of host
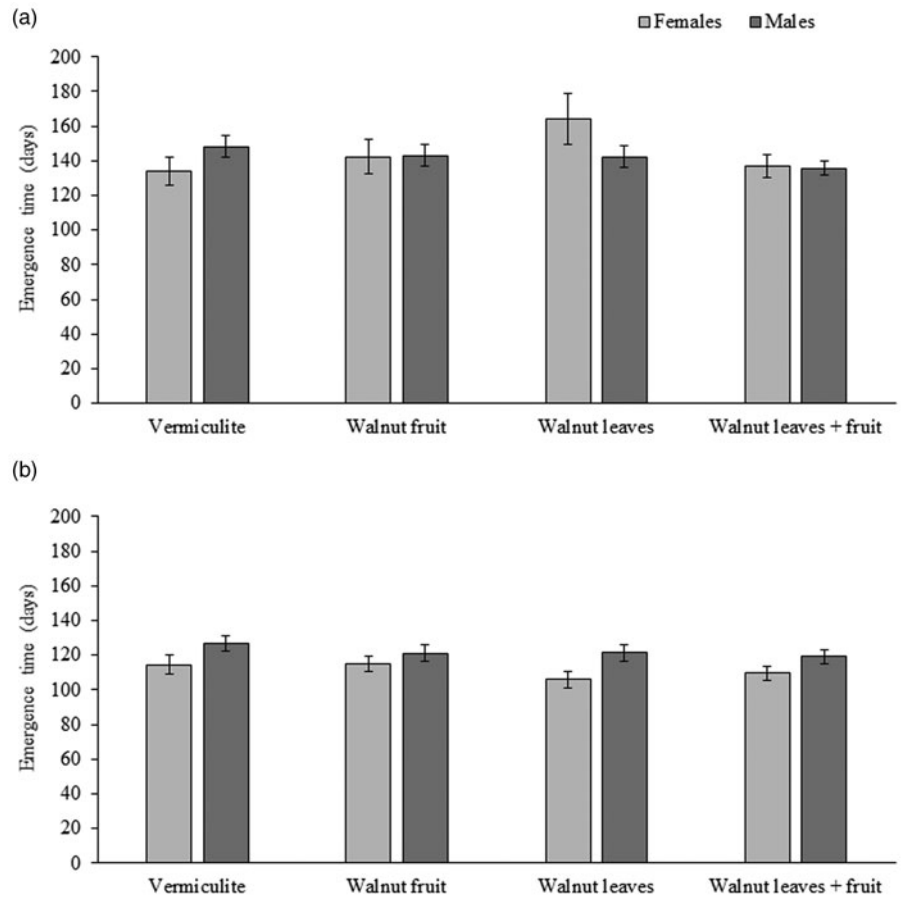
Figure 2. Mean days (± SE) to adult emergence of (a) *Rhagoletis completa* (N = 262 males, 124 females) (b) *R. zoqui* (N = 281 males, 242 females) in reference to pupae exposed to vermiculite alone or vermiculite with a fruit, leaves or leaves plus a fruit.
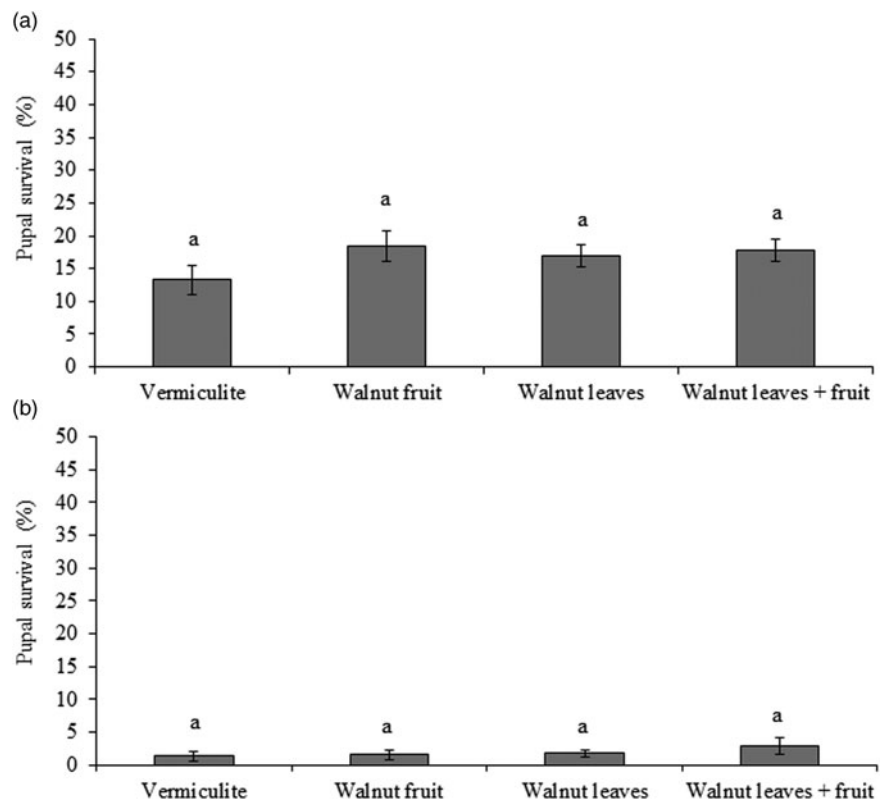
Figure 3. Mean pupae (± SE) survival of (a) *Rhagoletis completa* (N = 1800) (b) *R. zoqui* (N = 1800) in reference to pupae exposed to vermiculite alone or vermiculite with a fruit, leaves or leaves plus a fruit. Similar letters above bars indicate no-significant differences (P < 0.05).

plant parts varied between 1.3% and 2.8%, and there was no significant difference among treatments ($\chi^2 = 3.25$; df = 3; $P = 0.34$) (fig. 3b).

Parasitoids

A total of 104 parasitoids emerged from cups with *R. completa* pupae. Of these 104 parasitoids, 51 were identified as *Anagaspis alujai* (Wharton & Ovruski) and 53 were classified as *Diachasmimorpha* spp. All specimens of *A. alujai* parasitoids were females whereas *Diachasmimorpha* parasitoids were composed of *Diachasmimorpha juglandis* (Muesebeck) (27 adults) and *Diachasmimorpha mellea* (Gahan) (24 adults × 12 females and × 12 males). In the case of *D. juglandis*, the sample was damaged so the sex of individuals could not be accurately determined, however, a sub-sample not included in the experiment yielded a male biased sex ratio of 2.57 males per female. *Diachasmimorpha* species were identified at the end of the experiment and therefore emergence and mean duration of dormancy were evaluated together for both species. Mean percentage parasitism on total pupae varied between 4% and 8% and there were no significant differences among treatments ($F = 1.74$; df = 3,32; $P = 0.179$). When parasitism was considered, in reference to the total emerged adult flies, it reached 21% (104 parasitoids form 490 *R. completa* flies). Parasitoid species found on *R. completa* entered a dormancy of longer duration than that of their fly host.

Among 45 cups of fly pupae, parasitoids in the genus *Diachasmimorpha* emerged form 27 cups whereas *A. alujai* only emerged from eight cups. No emergence of *A. alujai* was observed in cups of the treatment that contained vermiculite + walnut leaves which was excluded from analyses. No significant differences were observed in the meantime to adult emergence of *A. alujai* or *Diachasmimorpha* for pupae subjected to different fly host plant parts ($\chi^2 = 2.15$; df = 2; $P = 0.340$) ($F = 0.183$; df = 3,49; $P = 0.907$) respectively. The mean time to adult emergence for *A. alujai*, was 260 ± 54 days (mean ± SD) and was significantly longer than that recorded for *Diachasmimorpha* parasitoids (216 ± 39 days (mean ± SD)), ($U = 365$, $P < 0.001$). As expected, both parasitoid species emerged significantly later than their fly host.

Only one parasitoid was reared from all *R. zoqui* pupae in the experiment and it was identified as female of *A. alujai*.

Exposure to juglone

Adult emergence of R. zoqui *pupae*

Out of 2520 pupae used for the juglone experiment, only 17 *R. zoqui* adults (0.67%) emerged from cups during the pre-winter period (35 days) without becoming dormant. No parasitoids emerged from any *R. zoqui* pupae recovered in 2018.

After the pre-winter period, a total of 272 *R. zoqui* adult flies emerged from all pupae reared in the experiment (84 cups, 30 pupae each one), with 131 males (48%) and 141 females (52%). Significant differences were observed in percent *R. zoqui* adult emergence for pupae exposed to juglone treatments or a non-treated vermiculite control ($F = 5.78$; df = 5146; $P < 0.001$) (fig. 4a). There was no statistically significant effect of sex ($F = 0.273$; df = 1146; $P = 0.602$) or of the interaction between sex and treatment ($F = 0.379$; df = 5146; $P = 0.862$). Exposure to two consecutive time periods under juglone treatment resulted in lower percent emergence of flies (fig. 4a).
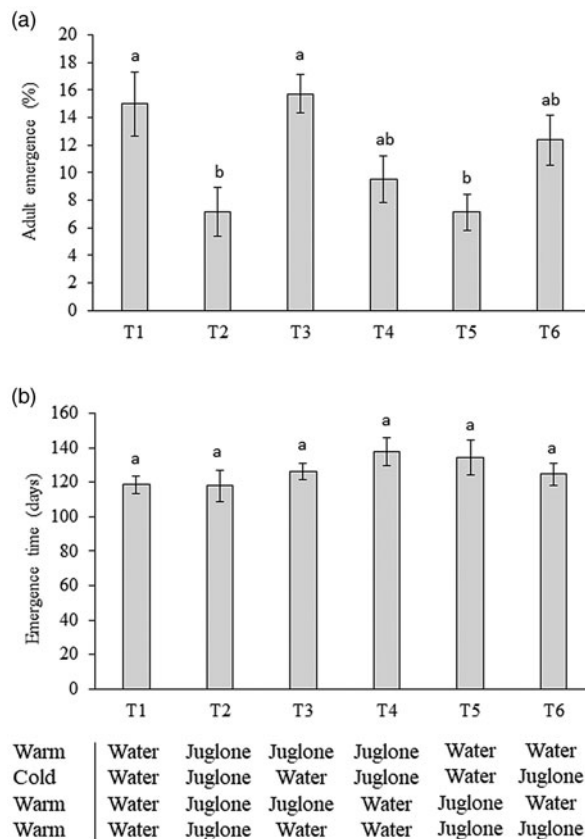
Figure 4. (a) Mean (± SE) percent emerged fly adults (males in dark grey bars followed by females in clear grey bars) ($N = 131$ males, 141 females), (b) mean days (± SE) to adult emergence of *R. zoqui* in reference to pupae exposed to vermiculite alone (water) or vermiculite treated with juglone over four 4-week time periods (warm 24°C and cold 5°C), following treatments indicated in table 1. Different letters above bars indicate significant differences ($P < 0.05$), Tukey HSD.

Emergence time of R. zoqui *pupae*

The mean emergence time of *R. zoqui* adults (excluding non-dormant flies) was not statistically different among pupae subjected to different juglone treatments ($F = 1.278$; df = 5,242; $P = 0.274$) (fig. 4b). The mean emergence time for males (mean ± SE) (122.1 ± 4.7 days) was not statistically different than that of females (117.1 ± 4.6 days) ($F = 0.941$; df = 1242; $P = 0.333$). No significant interaction between sex and treatment was observed ($F = 0.644$; df = 5242; $P = 0.666$) (fig. 4b).

Discussion

No, or very small proportions of *R. completa* and *R. zoqui* emerged as adults without becoming dormant. By contrast, a proportion of *R. completa* and a smaller proportion of *R. zoqui* pupae engaged in prolonged dormancy. Exposure to host plant parts (leaves and/or fruit) or juglone affected overwintering survival but not the duration of dormancy of *R. completa* and *R. zoqui*. During the host plant part exposure experiment, only overwintering pupae of *R. completa* exposed to fruit in vermiculite survived (emerged as adults) in greater proportion than *R. completa* or *R. zoqui* exposed to leaves and the control. In the case of exposure to juglone, at the tested concentration, regimes under prolonged exposure (two consecutive 4-week periods or more) caused mortality of overwintering *R. zoqui*. Overall, we failed to gather conclusive evidence indicating that host plant derived cues or juglone

are used as a signal (token stimulus) by walnut infesting *Rhagoletis* for regulation or fine tuning of dormancy.

Proportions of non-dormant flies and prolonged dormancy

While most species of *Rhagoletis* are considered to be univoltine (Bush, 1966; Boller and Prokopy, 1976), there is a proportion of the population that emerges as adults without becoming dormant. The proportion of non-dormant individuals among *Rhagoletis* can range from 53% to less than 1%, (Boyce, 1931; Rull *et al.*, 2016; Neven and Yee, 2017). For *R. pomonella*, total forgoing of dormancy can be induced by manipulating pre-winter environmental conditions (Prokopy, 1968). There is a latitudinal cline in the proportion of non-dormant individuals among North-American *R. pomonella*, with populations at northern latitudes being more prone to produce second generation individuals than those at lower latitudes (Dambroski and Feder, 2007). The latitudinal cline holds true for Mexican populations at the southern extreme of the distributional range of this species (Rull *et al.*, 2016), and appears to include *R. cingulata* (Rull *et al.*, 2018) which forgo dormancy in proportions under 1%. In the case of *R. completa*, AliNiazee and Boyce (1931) reported up to 75% and 53% of non-dormant individuals while Rull *et al.* (2019a) and our current results failed to detect any non-dormant individuals for the Mexican population in this species. Similarly, very few *R. zoqui* individuals forgo dormancy (Rull *et al.*, 2016). Overall, it seems that ecological conditions at the southern extreme of the genus do not favour persistence of this trait. This finding has negative implications from an applied perspective because it complicates selection of non-dormant strains necessary for development of continuous rearing.

As found for other members of the genus *Rhagoletis* (Boyce, 1931; Dean, 1973; Moraiti *et al.*, 2014; Rull *et al.*, 2018) a proportion of Mexican populations of *R. completa* and *R. zoqui*, engaged in prolonged dormancy. It has been proposed that prolonged dormancy in *R. cerasi*, evolved as a bet edging mechanism to cope with unpredictable environmental variability (Moraiti *et al.*, 2014). Plants in the genus *Juglans* are known to engage in mast seeding events which result in seasons with abundant fruit followed by seasons with scarce fruit (Stapanian and Smith, 1978; Kelly and Sork, 2002). Such recurrent inter annual variation could explain the existence of prolonged diapause in walnut infesting *Rhagoletis*.

Host plant part effects on survival and duration of dormancy

Exposure to host plant fruit and/or leaves or juglone had little effect on the duration of dormancy of *R. completa* and *R. zoqui*. Only *R. zoqui* pupae continuously exposed to juglone (four consecutive 4-week periods), emerged as adults sooner than pupae exposed to other regimes. However, duration of dormancy for such pupae was not different from that recorded for the untreated control, and therefore we cannot conclude that host plant derived chemicals are used by walnut flies as environmental cues for fine tuning of dormancy.

When examining adult emergence, the presence of fruit, with and without leaves, enhanced emergence of *R. completa* in comparison with pupae exposed to walnut tree leaves only and the control (vermiculite). By contrast, fruit presence had no effect of adult *R. zoqui* emergence. Humidity has been found to be important for survival of overwintering *Rhagoletis* pupae, especially during the final stages of dormancy (Rull *et al.*, 2019b).

The positive effect of fruit presence could have been due to release of plant produced chemicals. However, it is also possible that fruit presence enhanced survival by increasing humidity in the pupation media through water release during fruit breakdown. Thus, it is possible that *R. completa* may be more susceptible to desiccation than *R. zoqui,* which could account for the difference in response when fruit was present in treatments between the two species.

Exposure to juglone

Toxic effects of juglone on phytophagous insects have been widely reported (Islam and Widhalm, 2020). Nevertheless, some phytophagous species have been shown to be tolerant to this compound, yet at high concentrations it can still produce toxic effects (Thiboldeaux *et al.*, 1994). When exposing *R. zoqui* pupae to juglone, continuous application during two consecutive 4-week periods resulted in a reduction of adult emergence in comparison to other treatments and the control. Juglone concentration in our study was established on records found in soil under the canopy of *J. nigra* trees (de Scisciolo *et al.*, 1990), which is known to have the highest juglone concentration among studied species of *Juglans* (Willis, 2000) and is a natural host of *R. completa* (Yee *et al.*, 2014). Additionally, our methodology involved moistening vermiculite containing pupae on a weekly basis. Because we did not estimate the rate at which juglone degraded in cups, it could be that the compound accumulated at higher concentrations than 3 µg per g of vermiculite over the course of the experiment. In any case, it appears that rather than functioning as an environmental cue, at the tested concentration, juglone had a toxic effect on *R. zoqui*. Perhaps, juglone concentration under *J. pyriformis* trees, the native host of *R. zoqui* (Rull *et al.*, 2013), is normally lower than that under other species of *Juglans* trees and therefore the potential effect of juglone on regulation of dormancy of walnut infesting *Rhagoletis* should be tested at different lower concentrations than the one evaluated in this study.

Parasitism

As found in other studies involving Mexican populations of *R. completa* (Rull *et al.*, 2019a), we recorded 21% parasitism by three hymenopteran species (*A. alujai*, *D. mellea*, and *D. juglandis*). By contrast, parasitism on *R. zoqui* was almost non-existent, a surprising finding considering the fact that this fly species was collected within its native range and from its natural host plant. Because *A. alujai* did not emerge from fly pupae exposed to walnut leaves. it was not possible to reach conclusions on susceptibility of this parasitoid to host plant derived compounds such as juglone.

Conclusion

While our study yielded information on dormancy of Mexican populations of walnut infesting *Rhagoletis* and associated parasitoids, we failed to produce evidence indicating that juglone or host plant parts are used as a cue for fine tuning of dormancy in *R. completa* or *R. zoqui.* At the tested concentrations, juglone appeared to be toxic to exposed pupae. Lower concentrations of juglone should be evaluated to conclusively discard a potential effect, such tests should include *R. completa*. However, at present, juglone applications to the soil to disrupt synchrony of walnut infesting *Rhagoletis* and susceptible fruit are not supported by our data.

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