



# Effect of Pepper Variety on the Susceptibility of Pepper Weevil Parasitoids

Juan Eduardo Murillo-Hernández,<sup>1,2,✉</sup> J. Refugio Lomeli-Flores,<sup>1,5,✉</sup>  
Esteban Rodríguez-Leyva,<sup>1,✉</sup> Lauro Soto-Rojas,<sup>1</sup> Alfonso Torres-Ruiz,<sup>3</sup>  
Ma. Teresa Santillán-Galicia,<sup>1</sup> and Érica Muñiz-Reyes<sup>4</sup>

<sup>1</sup>Posgrado en Fitosanidad, Entomología y Acarología, Colegio de Postgraduados, Carretera México-Texcoco km 36.5, Montecillo, 56264 Texcoco, Estado de México, México, <sup>2</sup>INIFAP, Campo Experimental Tecomán, Carretera Colima-Manzanillo Km. 35, CP 28100 Tecomán, Colima, México, <sup>3</sup>Koppert México, Circuito el Marqués norte no. 82, Parque industrial El Marqués, CP 76246 El Marqués, Querétaro, México, <sup>4</sup>INIFAP, Campo Experimental Valle de México, Carretera Los Reyes-Texcoco km 13.5, Coatlinchán, CP 56250 Texcoco, Estado de México, México, and <sup>5</sup>Corresponding author, e-mail: [jrlomelif@hotmail.com](mailto:jrlomelif@hotmail.com)

Subject Editor: Juan Rull

Received 26 May 2022; Editorial decision 8 November 2022.

## Abstract

*Anthonomus eugenii* Cano (Coleoptera: Curculionidae) is a key pest of cultivated peppers (*Capsicum* species) in tropical and subtropical America. Here we evaluated the effect of five pepper varieties on the susceptibility of *A. eugenii* to the parasitoids *Bracon* sp. (Hymenoptera: Braconidae), *Eupelmus cushmani* (Crawford) (Hymenoptera: Eupelmidae), and *Jaliscoa hunteri* Crawford (Hymenoptera: Pteromalidae). Potential parasitism was estimated by comparative analysis of parasitoid ovipositor size and the depth to which host larvae develop inside the fruit. Highest potential parasitism rates were achieved by *Bracon* sp. and *E. cushmani* on árbol and habanero peppers (84–99%) while the lowest rates were achieved by *J. hunteri* on serrano, bell, and jalapeño (7–18%). To validate potential parasitism rates, the actual parasitism rate by *Bracon* sp. and *J. hunteri* on three varieties of peppers was assessed. Actual parasitism rates of *A. eugenii* larvae in árbol were similar for *Bracon* sp. and *J. hunteri* (33%), while on bell and jalapeño *Bracon* sp. achieved 24% and 13% parasitism and *J. hunteri* achieved 14% and 8%, respectively. In most cases, actual parasitism was lower than estimated potential parasitism, although the latter had a notable predictive power (predicted  $R^2 = 0.84$ ). Results showed that the host was more vulnerable on small-fruited varieties because larvae were closer to the pericarp and could be reached by parasitoid ovipositors; likewise, in varieties with little placenta and seed, some larvae fed in the pericarp, where they were more vulnerable.

**Key words:** pepper weevil, biological control, potential parasitism, actual parasitism rate

The pepper weevil, *Anthonomus eugenii* Cano (Coleoptera: Curculionidae), is the key pest of all cultivated peppers (*Capsicum* species) in tropical and subtropical America (Riley and King 1994). This species has been reported in the United States (Riley and King 1994, Ingerson-Mahar et al. 2015), Mexico (Laborde and Pozo 1984), Central America, and some Caribbean islands (Andrews et al. 1986). It is also an important greenhouse pest in southern Ontario, Canada (Fernández et al. 2017), and recently it was detected and subsequently eradicated from greenhouses in the Netherlands (Gaag and Loomans 2013) and Italy (Speranza et al. 2014).

Adults of *A. eugenii* cause damage when they feed and oviposit in flower buds or immature fruits; also, larvae cause fruit abscission when they feed on the placenta and seeds within fruits (Riley and King 1994, Toapanta et al. 2005). Production losses can reach 30–90% if control measures are not implemented in time (Riley and King 1994, Riley and Sparks 1995). Due to the biology of *A. eugenii*,

the most common methods used to prevent damage are either cultural, such as removing damaged fruit, or chemical via insecticide sprays against adults, which are the only stage exposed outside the fruit (Seal and Schuster 1995). It has been suggested that a natural enemy that attacks the immature stages could improve control of *A. eugenii* (Mariscal et al. 1998; Rodríguez-Leyva et al. 2007, 2012). However, to date, none of the parasitoids produced in the laboratory have been able to successfully control *A. eugenii* in the field (Schuster 2007; Rodríguez-Leyva et al. 2007).

Amongst the parasitoids that attack pepper weevils in nature, the one with most control potential is thought to be *Jaliscoa* (= *Catolaccus*) *hunteri* Crawford (Hymenoptera: Pteromalidae) (Gibson 2013). This species is one of the most common and abundant parasitoids attacking *A. eugenii* in the southern United States and Mexico (Riley and Schuster 1992; Rodríguez-Leyva et al. 2007, 2012). *Eupelmus cushmani* (Crawford) (Hymenoptera: Eupelmidae)

is also a common parasitoid of this pest throughout Mexico (Rodríguez-Leyva et al. 2007, 2012). In addition, one species in the genus *Bracon* (Hymenoptera: Braconidae), which is larger than (Fabales: Fabaceae) the previous two species mentioned, has been reported attacking *Anthonomus* spp. in alfalfa [(*Medicago sativa* L.)] in Guanajuato, Mexico. Although collected from other *Anthonomus* species, this *Bracon* sp. has been shown to parasitize and develop in *A. eugenii* on pepper fruits under laboratory and greenhouse conditions (A. Torres-Ruiz, unpublished data). These three species are all solitary, generalist, and sinovigenic ectoparasitoids that can develop on third instar larvae of *A. eugenii* and fourth instar larvae of the factitious host *Callosobruchus maculatus* F. (Coleoptera: Bruchidae) (Rodríguez-Leyva et al. 2000, 2012; JC Velázquez, Koppert Mexico, personal communication).

Plants can mediate interactions between phytophagous insects and their natural enemies (Price et al. 1980, Chen et al. 2015). Thus, the efficiency of parasitoids in controlling a pest could be associated with characteristics of the host plant, or its varieties, that have developed as a result of the domestication process (Wang et al. 2009, Chen et al. 2015). Some of these modifications could change the ratios of volatile substances released, resulting in a decrease in attraction of natural enemies (Köllner et al. 2008, Gols et al. 2011, Chen et al. 2015). An increase in the size of plant structures, such as fruits and seeds, can also decrease parasitoid efficiency if it affects host accessibility (Wang et al. 2009, Chen et al. 2015). This study was based on the hypothesis that thickness of the fruit wall (as determined by the variety and the quantity of placenta) and parasitoid ovipositor length were factors that determined parasitism rate on *A. eugenii*. The objective of this study was to determine the effect of pepper varieties on parasitism rates of three species of parasitoids on *A. eugenii*.

## Material and Methods

### Insect Colonies

A colony of *A. eugenii* was established at the Colegio de Postgraduados, Texcoco, Estado de Mexico (19° 30'20" N; 98° 52'55" O), on fruits of bell pepper following the methodology described by Rodríguez-Leyva (2006) in a bioclimatic chamber at 27 ± 5°C, 60 ± 10% RH, and a photoperiod of 12:12 (L:D) h. The parasitoid species were: *J. hunteri*, *E. cushmani*, and *Bracon* sp. All were reared on the bruchid beetle *C. maculatus* in chickpea using the methodology of Vasquez et al. (2005). The first two species originated from laboratory colonies maintained at the Colegio de Postgraduados; the third was provided by Koppert Mexico.

### Experimental Setup

#### Identification and Voucher Specimens

Identification of the *Bracon* species was attempted but failed because no recent taxonomic review of the group is available (Personal communication Alejandro Zaldívar, Institute of Biology, UNAM, Mexico). However, DNA extractions were made and the COI consensus sequences generated have been deposited in the GenBank database. The access numbers of specimens of *Bracon* sp. are MN449357, MN449358, MN449359, MN449360, MN449361, MN449362, MN449363, MN449364, MN449365, MN449366, MN449367, and MN449368. Voucher specimens of the three species were deposited in the Colegio de Postgraduados Insect Collection (CEAM-Hy-010).

#### Assay 1. Potential Parasitism Rates of *A. eugenii* by Three Parasitoid Species on Five Pepper Varieties

The efficacy of each parasitoid species on *A. eugenii* developing in different varieties of peppers was estimated by comparing their

potential parasitism rates. Hypothetically, a parasitoid can parasitize a larva when: the length of its ovipositor is equal to, or greater than, the depth at which the larvae develop within the fruit; potential parasitism rates were estimated based on measurements of these two parameters.

#### Depth and Distribution of *A. eugenii* Larvae in Pepper Fruits Collected from the Field

We sampled fruit from five commercial varieties of pepper: four varieties of *Capsicum annum* L. (Solanales: Solanaceae) (árbol, jalapeño, serrano, bell pepper), and one variety of *Capsicum chinense* Jacq (Solanales: Solanaceae) (habanero). Fruits with signs of *A. eugenii* infestation from all varieties were randomly collected from commercial plots during 2016 [árbol from Rosamorada, Nayarit (22° 06'45.3" N 105° 12'35.3" W); habanero from Ich-Ek, Campeche (19° 43'57.7" N 89° 57'38.8" W); jalapeño and serrano from Altamira, Tamaulipas (22° 25'53.5" N 97° 54'39.8" W); and bell pepper from El Marqués, Querétaro (20° 33'23.5" N 100° 15'58.4" W)]. Fruits were measured individually; the maximum length and diameter were captured using a digital Vernier (Stanley, 150 mm) and subsequently each one was dissected longitudinally. Fruits infested with *A. eugenii* larvae were handled carefully, so as not to move the larvae from their original position. One hundred images were obtained for each pepper variety using a scanner (hp SCANJET 5590) at a resolution of 600 pixels per inch. Then, the distance from pericarp to larva (=depth at which third instar larvae of *A. eugenii* were found inside the fruit) was measured using the free software ImageJ 1.48k. This larval stage, size about 3.3 mm with range 2.2–5.0 mm, was selected because it is the stage most susceptible to parasitism by the three parasitoid species evaluated. The within-fruit longitudinal distribution of larvae in each pepper variety was also described in relation to their basal (next to the calix), middle, or apical position.

#### Ovipositor Length of Parasitoids

The ovipositor length of each parasitoid species was measured following the methodology of Gómez-Domínguez et al. (2012); 35 females of each species were selected randomly from the laboratory colonies and placed in 70% alcohol. The ovipositor of each female was removed using entomological forceps and a dissection needle, and placed individually on a slide. A picture of each ovipositor was taken using a light microscope (Tessovar Carl Zeiss) equipped with a digital camera (PaxCam 3). Subsequently, the length of each ovipositor was measured from its base to the tip, using the free software ImageJ 1.48k.

#### Statistical Analysis

The length and diameter of pepper fruits, depth of *A. eugenii* larvae within fruits, and ovipositor lengths were compared by analysis of variance (ANOVA). In the case of larval depth, the data were transformed to log 10 so that they fulfilled the assumptions required by the parametric test. Subsequently, multiple post hoc comparisons of means were done using Tukey's tests ( $\alpha = 0.05$ ). The analyses were done using the R program (R Core team 2018).

To obtain a robust confidence interval for potential parasitism rates, a bootstrap analysis was conducted with replacement, contrasting ovipositor sizes versus depths at which larvae were found in each pepper variety, to produce a bootstrapped mean and 95% confidence interval based on 100,000 bootstrap samples. Unrestricted random sampling was used and observations in the data set were assumed to be independent. The analyses were done using the R program (R Core team 2018).

## Assay 2. Actual Parasitism and Host Feeding by Two Parasitoid Species on *A. eugenii* Larvae

Using information from the previous assay, actual parasitism rates of two parasitoid species were evaluated against *A. eugenii* on three pepper varieties. The varieties were selected because they represented contrasting depths at which *A. eugenii* could develop. The two parasitoid species evaluated were *J. hunteri* and *Bracon* sp., which had contrasting ovipositor lengths. Parasitoids were all 10-d-old mated females with oviposition experience. Fruit infestation and assays were done at  $27 \pm 5^\circ\text{C}$ ,  $60 \pm 10\%$  RH, and a photoperiod of 12:12 (L:D) h regime.

### Pepper Fruits and Infestation by *A. eugenii*

Immature pepper fruits of árbol ( $\text{Ø} = 1.25 \pm 0.01$  cm), jalapeño ( $\text{Ø} = 2.27 \pm 0.01$  cm), and bell pepper ( $\text{Ø} = 5.48 \pm 0.03$  cm) were obtained from pepper plants grown in 15 L polyethylene bags containing a porous volcanic gravel (tezontle; Substratos Agrícolas SAMG) as a growth substrate. The plants were irrigated daily with nutrient solution (Ultrazol 18-6-18 at 1 g/l) using an automated irrigation system, and maintained in a low-tech greenhouse at  $25 \pm 12^\circ\text{C}$ ,  $50 \pm 20\%$  RH, and the natural ambient photoperiod at Texcoco, Estado de Mexico ( $19^\circ 27' 41''$  N,  $98^\circ 54' 32''$  W).

Transparent plastic containers 3.8 L ( $23 \times 14 \times 14$  cm) were used both for fruit infestation and later as experimental arenas. Each container had two side holes ( $13 \times 8$  cm) covered with mesh fabric to allow ventilation. The opening of the container ( $\text{Ø} = 11$  cm) was sealed using a mesh fabric sleeve through which material could be introduced. Three to five pepper fruits of the same variety and two adult female *A. eugenii* per fruit were placed in each container for 24 h. Subsequently, the number of oviposition plugs (each one represented an egg that had been laid) on each fruit were counted and marked under a stereomicroscope using a permanent marker. The fruits selected for the assay had different levels of infestation depending on variety: 1–3 eggs in árbol; and 1–5 eggs in jalapeño and bell peppers. This level of infestation was selected because it allowed larvae to develop optimally inside each pepper variety. The development time of *A. eugenii* was estimated from the study of Toapanta et al. (2005), and all infested fruits were maintained in a bioclimatic chamber ( $27 \pm 2^\circ\text{C}$ ,  $60 \pm 10\%$  RH, a photoperiod of 12:12 (L:D) h regime) until larvae reached the third instar (7 d).

### Experimental Parasitism Rates

Pepper fruits infested with third instar larvae of *A. eugenii* and a female parasitoid (*J. hunteri* or *Bracon* sp.), were enclosed in each arena (plastic container). Mature mate females (5–8 day old) with oviposition experience and unfed were used in all the trials. Because

each fruit had different levels of infestation, the number of fruits in each arena was adjusted (5–10 for árbol pepper and 3–8 for jalapeño and bell peppers) to ensure 15–20 *A. eugenii* larvae were available in all arenas regardless of pepper variety. Infested fruits and parasitoids remained in each arena for 72 h and then the parasitoids were removed. A control treatment (without parasitoids) was included for each pepper variety to assess *A. eugenii* mortality due to manipulation. After 10 d, fruits were dissected and the number of parasitized larvae and larvae with signs of parasitoid host feeding (collapsed and/or with sting scars) were recorded using a stereomicroscope. A completely randomized experimental design was applied, within a  $3 \times 2$  factorial arrangement; each treatment was replicated ten times and the entire experiment was done on three occasions, each during a different week (total 30 replicates per treatment). Percentage parasitism and host feeding were calculated.

### Statistical Analysis

Parasitism was analyzed using a generalized linear model (Proc GLM) with a binomial distribution and logit link function (logistic regression). The response variables were the proportions of parasitized larvae, larvae with indications of parasitoid host feeding, and the sum of parasitism plus host feeding. A logistic regression model was adjusted for each response variable, the effect of the two parasitoid species, three pepper varieties, and their interaction were analyzed. When there was more variation than could be explained by the assumed probability distribution, we tested the ratio of the treatment mean deviance to the residual mean deviance against the F-distribution, rather than testing the usual treatment deviance against the  $\chi^2$  distribution. There was no mortality in the controls which were not included in the analysis. Potential and actual parasitism rates were fitted to a regression model to obtain the predicted  $R^2$ . The analyses were done using the R program (R Core team 2018).

## Results

### Assay 1: Potential Parasitism Rates of *A. eugenii* by Three Parasitoid Species on Five Pepper Varieties Depth and Distribution of *A. eugenii* Larvae in Pepper Fruits Collected from the Field

Pepper fruits varied significantly amongst varieties in diameter ( $F_{4, 495} = 549.68$ ;  $P < 0.0001$ ) and length ( $F_{4, 495} = 74.24$ ;  $P < 0.0001$ ). Bell peppers had the largest diameter ( $40.4 \pm 1.0$  mm), followed in descending order by habanero, jalapeño, serrano, and finally árbol, which had the smallest diameter (Table 1). The variety serrano had the greatest length ( $51.2 \pm 1.0$  mm) followed in descending order by jalapeño, bell pepper, árbol, and finally habanero (Table 1).

**Table 1.** Size of fruit from five varieties of pepper in millimeters (mean  $\pm$  SE) and distribution of *Anthonomus eugenii* larvae within them

Variety	n	Length	Diameter	Distribution of <i>A. eugenii</i> larvae (%) in basal, middle, and apical sections of fruit		
				Basal	Central	Apical
Árbol	100	38.4 $\pm$ 1.3C	8.0 $\pm$ 0.1E	37 $\pm$ 0.05	47 $\pm$ 0.05	16 $\pm$ 0.04
Habanero	100	26.4 $\pm$ 0.5D	23.5 $\pm$ 0.4B	33 $\pm$ 0.05	21 $\pm$ 0.04	46 $\pm$ 0.05
Jalapeño	100	45.2 $\pm$ 1.1B	20.2 $\pm$ 0.3C	46 $\pm$ 0.05	43 $\pm$ 0.05	11 $\pm$ 0.03
Bell	100	39.3 $\pm$ 1.3C	40.4 $\pm$ 1.0A	51 $\pm$ 0.05	28 $\pm$ 0.05	21 $\pm$ 0.04
Serrano	100	51.2 $\pm$ 1.0A	17.6 $\pm$ 0.2D	71 $\pm$ 0.05	15 $\pm$ 0.04	14 $\pm$ 0.03

Means in the same column and followed by the same capital letter are not significantly different to each other statistically (Tukey  $P < 0.05$ ).

Larvae of *A. eugenii* varied in their distribution within the fruits of each variety of pepper collected from the field. In árbol, 84% of larvae were found in the basal and middle thirds of the fruit, while in habanero larvae were found in the apical and basal thirds of the fruit (81%). In jalapeño 89% of larvae were found in the basal and middle thirds, with fewer in the apical third. In bell pepper the largest number of larvae (51%) was found in the basal third of the fruit with fewer in the middle and apical thirds. In serrano 71% of larvae were found in the basal third with smaller numbers in the middle and apical thirds (Table 1).

The depth at which larvae of *A. eugenii* were located within fruits varied significantly depending on variety ( $F_{4,495} = 64.67$ ;  $P < 0.0001$ ). In árbol and habanero they were found nearer the fruit surface:  $1.42 \pm 0.05$  and  $1.72 \pm 0.17$  mm, respectively. In serrano, larvae of *A. eugenii* were located at greater depth than in árbol and habanero, but not as deep as in jalapeño and bell pepper where they were three times deeper than in the árbol and habanero (Fig. 1).

There were significant differences in ovipositor length amongst parasitoid species ( $F_{2,102} = 601.35$ ;  $P < 0.0001$ ). *Bracon* sp. had the longest ovipositor ( $4.28 \pm 0.05$  mm; range 3.54–4.80 mm), while *J. hunteri* had the shortest ( $1.93 \pm 0.03$  mm; range 1.43–2.50 mm), at less than half the length of the ovipositor of *Bracon* sp., and shorter than the ovipositor of *E. cushmani* ( $3.33 \pm 0.06$  mm; range 2.19–3.79 mm) (Fig. 2). In general, potential parasitism rates on *A. eugenii* larvae depended on the length of the parasitoid ovipositor, and the depth at which third instar *A. eugenii* larvae developed in each pepper variety. The parasitoid with the greatest potential parasitism rate was *Bracon* sp. (Table 2), and there were significant differences between *Bracon* sp. and *E. cushmani* and between *Bracon* sp. and *J. hunteri*, as well as differences between *E. cushmani* and *J. hunteri*.

Considering the infested peppers fruits collected from the field: potentially, between 84.41 and 99.90% of *A. eugenii* larvae developing in árbol and habanero peppers would be susceptible to

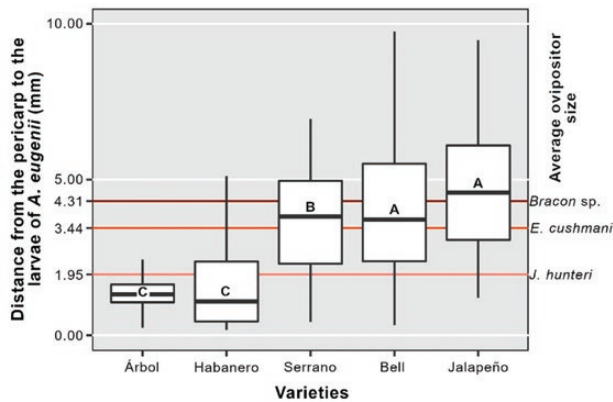


Fig. 1. Depth at which *Anthonomus eugenii* larvae were found within naturally-infested field-collected pepper fruits. Means with the same capital letter are not significantly different to each other (Tukey,  $\alpha = 0.05$ ).



Fig. 2. Comparison of ovipositor length of three pepper weevil parasitoids.

parasitism by *Bracon* sp. and *E. cushmani*, and to a lesser extent by *J. hunteri* (71.47–84.82%). For the three parasitoid species, the least parasitism would be expected in serrano, bell, and jalapeño peppers. Potential parasitism rates by *Bracon* sp. in bell and jalapeño varieties would be expected to be 45.54–54.00%, *E. cushmani* could reach 31.13–38.17% parasitism, while for *J. hunteri* it would not be expected to exceed 7.22–18.75% (Table 2).

## Assay 2: Actual Parasitism and Host Feeding (=Total Mortality) by Two Species of Parasitoids on *A. eugenii* Larvae

Parasitism of third instar larvae of *A. eugenii* was significantly influenced by parasitoid species ( $F_{1,174} = 6.27$ ,  $P = 0.012$ ), by pepper varieties ( $F_{2,174} = 44.99$ ,  $P < 0.0001$ ), and by the interaction of both factors ( $F_{2,174} = 7.23$ ,  $P = 0.027$ ).

Parasitism of *J. hunteri* compared to *Bracon* sp. on árbol pepper had no statistical difference (odds ratio 1.02:1.00;  $F_{1,116} = 0.58$ ,  $P = 0.447$ ). In contrast, in jalapeño pepper parasitism by *J. hunteri* is less likely than parasitism by *Bracon* sp. (odds ratio 0.57:1.00;  $F_{1,116} = 3.29$ ,  $P = 0.069$ ); similar results were obtained in bell pepper (odds ratio 0.54:1.00;  $F_{1,116} = 6.32$ ,  $P = 0.012$ ).

For *Bracon* sp. parasitism on jalapeño pepper was less likely than on árbol pepper (odds ratio 0.34:1.00;  $F_{1,116} = 83.58$ ,  $P < 0.0001$ ); furthermore, this parasitoid was less likely to parasitize on bell compared to árbol (odds ratio 0.61:1.00;  $F_{1,116} = 40.70$ ,  $P < 0.0001$ ). Finally, parasitism on bell was more likely than on jalapeño (odds ratio 1.82:1.00;  $F_{1,116} = 13.44$ ,  $P = 0.0002$ ).

Parasitism of *J. hunteri* was influenced by variety, comparing the relative probability of parasitism on jalapeño versus árbol (odds ratio 0.19:1.00;  $F_{1,116} = 3.29$ ,  $P = 0.069$ ). This parasitoid is less likely to parasitize on bell than on árbol (odds ratio 0.32:1.00;  $F_{1,116} = 6.32$ ,  $P = 0.012$ ). Additionally, *J. hunteri* was more likely to parasitize on bell compared to jalapeño although the differences were not significant (odds ratio 1.74:1.00;  $F_{1,116} = 0.018$ ,  $P = 0.892$ ).

Host feeding by *J. hunteri* compared to *Bracon* sp. in árbol pepper had no statistical difference (odds ratio 1.40:1.00;  $F_{1,116} = 1.02$ ,  $P = 0.313$ ). In contrast, host feeding by this parasitoid was modified as a function of variety; e.g., in jalapeño pepper, host feeding by *J. hunteri* is less likely than by *Bracon* sp. (odds ratio 0.82:1.00;  $F_{1,116} = 4.01$ ,  $P = 0.045$ ); on the other hand, host feeding by both species was similar in bell pepper ( $F_{1,116} = 1.82$ ,  $P = 0.177$ ).

For *Bracon* sp. host feeding in jalapeño was less likely than in árbol (odds ratio 0.70:1.00) with significant differences ( $F_{1,116} = 24.37$ ,  $P < 0.0001$ ); moreover, this parasitoid was less likely to host feeding in bell pepper than in árbol (odds ratio 0.60:1.00;  $F_{1,116} = 33.31$ ,  $P < 0.0001$ ). Finally, host feeding in bell pepper was as likely as on jalapeño (odds ratio 0.86:1.00;  $F_{1,116} = 0.15$ ,  $P = 0.695$ ).

For the parasitoid *J. hunteri*, comparing the relative probability of host feeding in jalapeño versus árbol, the interaction of parasitoid and variety is significant (odds ratio 0.41:1.00;  $F_{1,116} = 4.01$ ,  $P = 0.045$ ). *J. hunteri* recorded less host feeding in bell pepper than in árbol (odds ratio 0.43:1.00), although the differences were not significant ( $F_{1,116} = 0.15$ ,  $P = 0.695$ ). Additionally, *J. hunteri* recorded a similar host feeding probability in bell pepper and jalapeño ( $F_{1,116} = 0.63$ ,  $P = 0.428$ ).

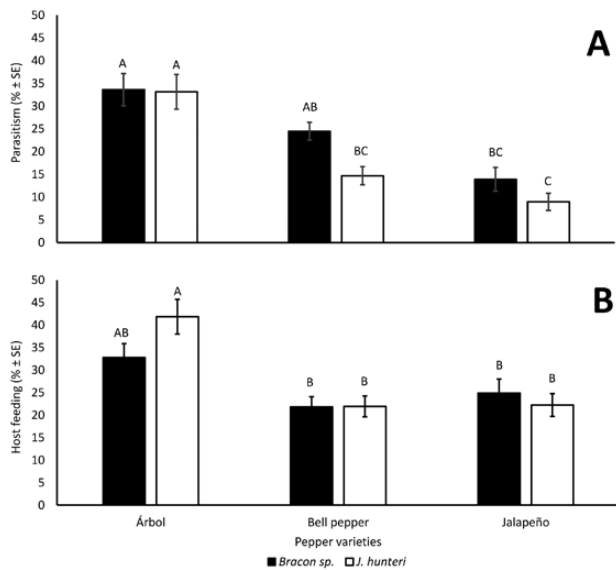
Total mortality of *A. eugenii* (the sum of parasitism plus host feeding) was not significantly affected by parasitoid species ( $F_{1,174} = 1.22$ ,  $P = 0.268$ ), but it was significantly affected by pepper variety ( $F_{2,174} = 90.92$ ,  $P < 0.0001$ ). Total mortality on árbol pepper reached 66–75% versus 31–46% in bell and jalapeño peppers. The interaction between factors indicates that *J. hunteri* achieved slightly higher

**Table 2.** Potential parasitism estimated from the comparison between ovipositor sizes versus depths at which larvae were found in each pepper variety

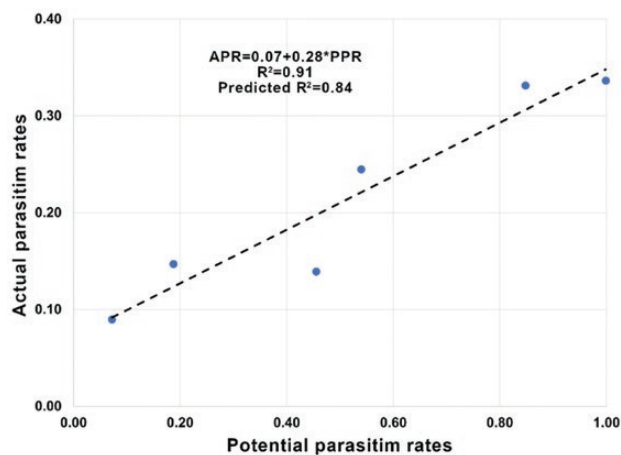
Variety	<i>Bracon</i> sp.		<i>E. cushmani</i>		<i>J. hunteri</i>	
	Mean <sup>a</sup> (%)	95% CI <sup>b</sup>	Mean (%)	95% CI	Mean (%)	95% CI
Árbol	99.89	99.88, 99.90	98.85	98.81, 98.88	84.82	84.71, 84.92
Habanero	89.60	89.51, 89.70	84.41	84.31, 84.52	71.47	71.33, 71.60
Jalapeño	45.54	45.40, 45.69	31.13	30.99, 31.27	7.22	7.14, 7.30
Bell	54.00	53.85, 54.16	38.17	38.02, 38.32	18.75	18.63, 18.86
Serrano	59.73	59.57, 59.88	38.88	38.73, 39.02	17.77	17.66, 17.89

<sup>a</sup>Potential parasitism rates were estimated by bootstrap analysis and the results were multiplied by 100 to obtain percentages.

<sup>b</sup>Confidence intervals (CIs) were based on 100,000 bootstrap samples. If CIs do not overlap, the difference is significant ( $P = 0.05$ ).



**Fig. 3.** Mortality by parasitism (A) and host feeding (B) (% ± SE) caused by two parasitoid species attacking third instar larvae of *Anthonomus eugenii* on three pepper varieties. Same letter above the bars indicates nonsignificant differences between treatments.



**Fig. 4.** Comparative analysis of potential parasitism rate (PPR) versus actual parasitism rate (APR).

mortality than *Bracon* sp. on árbol pepper, although no significant differences were recorded ( $F_{1,116} = 0.08$ ,  $P = 0.776$ ); additionally, *Bracon* sp. performed better than *J. hunteri* in bell ( $F_{1,116} = 11.52$ ,

$P = 0.0006$ ) and jalapeño peppers ( $F_{1,116} = 10.1552$ ,  $P = 0.0014$ ). The percentages of mortality by parasitism and host feeding are illustrated in Fig. 3, and it allows predicting the parasitism rates ( $R^2 = 0.84$ ) (Fig. 4) that could be expected for *Bracon* sp. (habanero: 32%, serrano: 24%) and *J. hunteri* (habanero: 27%, serrano: 12%).

## Discussion

This study used the length and diameter of pepper fruits as an indicator of the potential depth at which larvae of *A. eugenii* might be found; length was found to be an irrelevant variable. Fruit diameter was a better indicator of the likely distance between *A. eugenii* larvae and the fruit wall (=depth). This is explained, in part, by the biology and behavior of *A. eugenii* because larvae prefer to develop on seeds and immature placenta within the fruits, and only occasionally on the fruit wall (Riley and King 1994, Toapanta et al. 2005, Rodríguez-Leyva 2006).

The pepper variety with the largest diameter was bell pepper, followed in descending order by habanero, jalapeño, serrano, and árbol. Therefore, we expected that the depth of *A. eugenii* larvae within their fruits would follow the same order. However, this was not always the case. In jalapeño, bell, and serrano peppers *A. eugenii* larvae were observed at greater depth inside the fruits than in árbol and habanero. Larvae were found nearer the fruit surface in bell and habanero peppers than in jalapeño peppers, even though these varieties have a larger diameter than jalapeño; this corroborates the fact that larvae prefer to develop within fruits (placenta or pericarp), which influences their susceptibility to parasitism.

Female *A. eugenii* prefer to lay eggs in the basal third of fruits, usually near the base of the calyx, but after emerging from the egg, the larvae move inside the fruit to feed on the placenta and immature seeds (Toapanta et al. 2005, Rodríguez-Leyva 2006, Seal and Martin 2016). Even though the amount of placenta and the number of seeds were not quantified in this study, it was evident that the largest quantity of these tissues was found in the basal third of fruits, then in the middle with only a little in the apical third in bell, jalapeño, and serrano peppers. In addition, it has been shown that the highest levels of protein, reduced sugars, and capsaicin are in the placenta compared with the seeds and pericarp (Rodríguez-Leyva 2006, Simonovska et al. 2014). This indicates that larval distribution within fruits is influenced by the nutritional value of the tissues on which they feed and develop, and not their proximity to the fruit surface and potential risk of parasitism. This typical larval distribution was not apparent for those in árbol peppers, possibly due to their smaller diameter (the smallest of all varieties), and because the placenta and seeds are more evenly distributed throughout the fruit.

In the case of habanero peppers, the fruits have very little placenta and few seeds, sometimes none at all. This means that *A. eugenii* larvae have to develop in the wall of the fruit; the wall is thicker in the basal and apical sections which may account for the observed development in these sections. It must be remembered that the pepper fruits were infested naturally, and the adult population density of *A. eugenii* was not recorded at each collection site. Therefore, it is possible that population density is another contributory factor in the distribution of larvae in each pepper variety.

The parasitoid *Bracon* sp. had the longest ovipositor followed in descending order by *E. cushmani* and *J. hunteri*. Until now no experimental evidence has been presented on the role of parasitoid ovipositor length and host feeding habits on mortality of *A. eugenii*, or on how the biology of *A. eugenii* and pepper varieties might influence successful parasitism. However, the same hypothesis has been proposed and tested for other parasitoid species, specifically for fruit fly parasitoids (Sivinski and Aluja 2012). In fruit fly parasitoids, a positive correlation was found between the length of the ovipositor and parasitoid foraging; short-ovipositor parasitoids were rarely found foraging on large fruits (Sivinski and Aluja 2012).

Potential parasitism rates were a good predictor of actual parasitism rates. Also, they might help explain the variation in parasitism rates of *J. hunteri* on *A. eugenii* observed in bell and jalapeño peppers in the field (2–50%) (Riley and Schuster 1992; Rodríguez-Leyva et al. 2007, 2012). However, potential parasitism overestimated the actual performance of *Bracon* sp.; as the parasitoid with the longest ovipositor, it was expected to be the most effective parasitoid. This was not always the case, which is probably related not only to the size of the ovipositor, but also to other biological and behavioral characteristics of each parasitoid species.

Based on potential parasitism rates, we would expect that total mortality (i.e., actual parasitism plus host feeding) caused by *Bracon* sp. and *J. hunteri* would be greater in árbol peppers than in bell and jalapeño peppers because árbol peppers have the smallest diameter and *A. eugenii* larvae develop closer to the wall of the fruit than in bell pepper and jalapeño peppers. These clear trends in total mortality were what we observed. However, *J. hunteri* always achieved around 10% greater larval mortality in árbol peppers than *Bracon* sp. This difference could be attributed to the fact that ovipositor length was not an advantage for *Bracon* sp. in árbol peppers as it would have been in larger bell and jalapeño peppers.

In greenhouse assays, *J. hunteri* was more efficient at host location than *Bracon* sp. (unpublished data); this suggests that *J. hunteri* may have some competitive advantages over *Bracon* sp. It is well known that, despite being a generalist parasitoid, *J. hunteri* is the parasitoid most frequently found attacking *A. eugenii* in the field in the United States (Riley and Schuster 1992), Mexico (Rodríguez-Leyva et al. 2000, 2007; 2012) and Central America (Cross and Mitchell 1969; Gibson 2013). It is also reported from southern Ontario, Canada (Labbé et al. 2018). Furthermore, the *Bracon* sp. evaluated, which has not been identified, is a generalist parasitoid on other curculionid species on alfalfa, and has not been collected during any surveys of *A. eugenii* natural enemies in the field (Mariscal et al. 1998; Rodríguez-Leyva et al. 2007, 2012).

It should also be noted that host feeding by sinovigenic species of parasitoid was an important factor in overall mortality of *A. eugenii* larvae. This additive effect has been reported in other sinovigenic species where mortality due to host feeding was similar or greater than parasitism (Jervis and Kidd 1986, Bernardo et al. 2006, Cerón-González et al. 2014). In this regard, it was recently discovered that *J. hunteri* also feeds on first and second

instar larvae of *A. eugenii* and, theoretically, these developmental states are closer to the fruit wall (Murillo-Hernández et al. 2019). Thus, mortality determined in this study could be a slight underestimate of the actual mortality in the field, because we only used third instar larvae.

So far, no natural enemies of *A. eugenii* have been reported as providing significant levels of larval parasitism to achieve pest regulation when released as an inundative control agent (Riley and Schuster 1992, Rodríguez-Leyva et al. 2007, Schuster 2007). We provided evidence to suggest that mortality due to larval parasitism could be achieved by augmentative releases of parasitoids against *A. eugenii* and would be more likely to provide successful pest regulation: in pepper varieties with small diameters (e.g., árbol); where larvae develop in the pericarp (e.g., habanero), or on alternate hosts with small fruits [e.g., *Solanum americanum* Mill. (Solanales: Solanaceae)] as suggested by Schuster (2007). While a 35–40% mortality rate of *A. eugenii* in bell pepper fruits may not be considered sufficient for a parasitoid to be described as an ‘excellent’ natural enemy, it could still be an important tool within integrated management of this pest in protected agriculture. Future evaluations combining cultural and mechanical control with augmentative releases of *J. hunteri* under greenhouse conditions could offer a clearer idea of the potential of this control tool.

In conclusion, the distribution and distance between larvae of *A. eugenii* and the fruit wall (=depth) was influenced by the quantity and distribution of the tissue on which *A. eugenii* prefer to feed within pepper fruits; this varied amongst commercial varieties of pepper. The ovipositor length of parasitoids, such as *J. hunteri* and *Bracon* sp., could limit access to *A. eugenii* larvae and, therefore, successful parasitism as well as host feeding. We have provided experimental evidence to support the hypothesis that ovipositor length in parasitoids and fruit diameter, the latter of which has been modified by plant breeding, affects the accessibility of parasitoids to their host. Consequently, hosts are more vulnerable in smaller fruits.

## Acknowledgments

Thanks go to: Julio Velázquez-González, of Koppert Mexico, for providing biological material and facilities for this study; Jorge M. Valdez Carrasco for advice on taking and processing images; and Ariel W. Guzmán Franco for help with analysis and statistical advice. We also acknowledge the National Council of Science and Technology (CONACyT), Mexico, for the full doctoral scholarship granted to the first author. This manuscript is part of the doctoral thesis of the first author (JEMH) who received a full scholarship from CONACyT-México; also it was supported by the Colegio de Postgraduados and the National Council of Science and Technology (CONACyT), Mexico (grant numbers PM18-10-44, EFIDT 8080802).

## Author Contribution

JRLF, ERL and JEMH (Conceptualization), JRLF, ERL and ATR (methodology), JRLF, ERL, LSR, MTSG and EMR (validation), LSR and JEMH (formal analysis), JRLF, ERL and JEMH (investigation), JRLF, ERL and JEMH (writing—original draft preparation), JRLF, ERL, JEMH, LSR, ATR, MTSG and EMR (writing—review and editing), JRLF and ERL (supervision), ERL (project administration), JRLF and ERL (funding acquisition). All authors have read and agreed to the published version of the manuscript.

## Supplementary Data

Supplementary data are available at *Journal of Insect Science* online.

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