Natural enemies of *Anthonomus eugenii* (Coleoptera: Curculionidae) in Canada

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Abstract—The pepper weevil, *Anthonomus eugenii* Cano (Coleoptera: Curculionidae), is the most important pest of pepper (*Capsicum* Linnaeus; Solanaceae) crops in North America. Native to Mexico, the southern United States of America, and Central America, it is intercepted in Canada when peppers are imported to supplement domestic production. Given the proximity of greenhouse and field production to packing facilities, this pest poses a serious risk to the cultivation of peppers in Canada. Once established, it is difficult to control because immature stages of the weevil are protected within the pepper fruit. As such, chemical control targeting these life stages is not effective, and other strategies, including biological control, may prove useful. To explore the potential for biological control options to manage the pepper weevil in areas at risk in Canada, natural enemy surveys were conducted in southern Ontario following the reports of transient, localised field populations in 2016. Parasitoids belonging to three Hymenoptera families including Pteromalidae (*Jaliscoa hunteri* Crawford, *Pteromalus anthonomi* Ashmead), Eupelmidae (*Eupelmus pulchriceps* Cameron), and Braconidae (*Nealiolus* Mason species, *Bracon* Fabricius species) were reared from infested field-collected pepper fruits. Together, these new natural enemy records could facilitate the exploration and development of novel agents for the biological control of the pepper weevil.

Introduction

The pepper weevil, *Anthonomus eugenii* Cano (Coleoptera: Curculionidae) is a major pest of pepper crops, genus *Capsicum* Linnaeus (Solanaceae). First described by Donaciano Cano y Alcacio (Cano 1894), the pepper weevil is native to Central America, Mexico, and the southwestern United States of America (Clausen 1978). It has also spread to the Caribbean and French Polynesia, with sporadic occurrences in Italy, The Netherlands, Hawaii, and southern Canada (Nederlandse Voedsel-en Warenautoriteit 2013; Speranza *et al*. 2014). To meet market demand in Canada, peppers are often imported to supplement local production, generally from southern regions where the pepper weevil is widespread. These imports can be the source of subsequent outbreaks of the pest when retail locations and/or packing facilities are in close proximity to field or greenhouse pepper production (Canadian Food Inspection Agency 2011; Baker *et al*. 2012; Nederlandse Voedsel-en Warenautoriteit 2013). Similarly, movement of alternate Solanaceous hosts and/or packing materials from the established range of the pepper weevil can serve as potential sources of invasion (Costello and Gillespie 1993). In the United States of America, pepper weevil was recently identified as a significant pest as far north as New Jersey, likely the result of infested pepper shipments from the known range of the pest (Ingerson-Mahar *et al*. 2015). Through a similar pathway, the pepper weevil was reported in 1992 in southern British

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Columbia, Canada, but was eradicated shortly thereafter (Costello and Gillespie 1993). It was subsequently found in greenhouses in southern Ontario, Canada in 2009 and 2010; however, no regulatory action was taken (Canadian Food Inspection Agency 2011; Nederlandse Voedsel- en Warenautoriteit 2013). Although it is unclear whether pepper weevil is able to permanently establish and overwinter in Canada, repeated annual invasions may result in localised, transient populations that are able to cause significant damage to greenhouse and field peppers during a given growing season. Such an event occurred in 2016 in southern Ontario in field and greenhouse pepper crops (Fernández *et al*. 2017), and clearly demonstrates that this pest can have a serious impact in major pepper growing regions in Canada. Furthermore, rising global temperatures resulting from climate change may facilitate the establishment of species that were previously unable to survive the harsh winter climate (Hellmann *et al*. 2008), and this once transient pest problem may progress into a more permanent and global concern.

Management of the pepper weevil is based on scouting, physical pest exclusion, cultural practices (*i.e.*, removing and disposing of infested fruit), and chemical control of adults (Frantz and Mellinger 1998; Servín *et al*. 2002). Management tactics targeting the potentially more vulnerable immature life stages (*e.g.*, egg, larvae, and pupae) are generally not feasible because these life stages all occur within the protected environment of the pepper fruit. Thus, the adult weevil is the only life stage that can be targeted for management using pesticides. Concerns over the development of insecticide resistance and the undesired effects these have on beneficial arthropod communities has led to consideration of biological control as a means of pest management.

Documentation of releases of parasitoids that attack the pepper weevil has come from Mexico, the southern United States of America, and Hawaii. From 1934 to 1937, introductions of *Eupelmus cushmani* (Crawford) (Gibson 2016) (Hymenoptera: Eupelmidae) and *Jaliscoa hunteri* (Crawford) (Gibson 2013) (Hymenoptera: Pteromalidae) were performed in Hawaii and resulted in successfully established populations of the parasitoids for biological control of pepper weevil (Clausen 1978). Recently, there has been renewed interest in biological control of the pepper weevil, namely to assess the efficacy of *Triaspis eugenii* Wharton and López-Martínez (Hymenoptera: Braconidae) (Toapanta 2001; Rodríguez-Leyva 2006) and *J. hunteri* in field bell pepper in Florida, United States of America (Schuster 2012). In fact, Shuster (2012) demonstrated that *J. hunteri* significantly reduces the number of weevil-infested bell pepper fruit. Recent natural enemy surveys have documented a considerable diversity of parasitoids associated with the pepper weevil, and relatively little is known regarding their ecology and host range. Hymenopteran parasitoids from at least five families have been identified from the pepper weevil, including members of the Braconidae (*Urosigalphus* Ashmead, *Bracon* Fabricius, *Aliolus* Say, *Triaspis* Haliday), Eurytomidae (*Eurytoma* Illiger), Eulophidae (*Ceratoneura* Ashmead, *Baryscapus* Förster), Eupelmidae (*Eupelmus* Dalman), and Pteromalidae (*Jaliscoa* Bouček) (Cortez *et al*. 2005; Rodríguez-Leyva *et al*. 2007). Among these, Rodríguez-Leyva *et al*. (2007) found that *T. eugenii*, *Urosigalphus* species, and *J. hunteri*, accounted for 96% of all specimens collected in Mexico. To determine if parasitoids are associated with transient, seasonally established populations of the pepper weevil in southern Ontario, Canada a survey was conducted to identify the pepper weevil natural enemy community in this region.

Materials and methods

Collection of insects

Surveys were conducted weekly at multiple locations in southwestern Ontario from April to November 2016. Sampling began in Essex County, where the pepper weevil was first detected in fields beginning in April. As the pepper weevil range expanded in late summer, sampling was extended to Norfolk and Elgin Counties. In total, 10 locations were surveyed, including six conventional and one organic pepper fields, as well as two conventional and one organic pepper greenhouses.

Fruits that showed pepper weevil damage, including calyx yellowing, oviposition marks or premature fruit drop (Campbell 1924; Addesso *et al*. 2007) were collected from each location. Peppers belonging to six varieties of *Capsicum annuum* Linnaeus (Solanaceae) including hot

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cherry, banana, mini sweet, bell, jalapeño, and chili were collected as available. Also collected directly from plants were peppers of the scotch bonnet variety of *Capsicum chinense* Jacquin (Solanaceae), although no signs of oviposition or weevil emergence were detected from this variety. A total of 2604 aborted peppers were collected, with a range of sample sizes from each location as available. Collected peppers were maintained in a fine mesh bag sealed and held in a single pepper layer so as to reduce the decomposition of peppers within. All peppers were maintained in a controlled environment chamber set at 24 °C, 16 hour photoperiod, 50% relative humidity. Once a week, emerged weevils and natural enemies were collected and counted from each pepper batch. Peppers were monitored for up to five weeks, until no further adult weevils or parasitoids emerged for at least one week. Peppers were then cut open and checked for any remaining weevils or parasitoids before being discarded. Per cent parasitism was calculated for each batch of peppers collected ((# emerged parasitoids/# weevils + # emerged parasitoids) × 100). Emerged weevil and parasitoid specimens were preserved in 90% alcohol. Specimens were sent to the National Insect Identification Service, Agriculture and Agri-Food Canada, Ottawa, Ontario, where they were identified by taxonomic experts and deposited at the Canadian National Collection of Insects, Arachnids, and Nematodes.

Genetic characterisation of parasitoids

To contribute to the development of a DNA barcode database for parasitoids associated with the pepper weevil, the DNA barcode region of the mitochondrial cytochrome oxidase I (COI) gene was sequenced for representative specimens of each parasitoid species collected and for which vouchers were deposited at the Canadian National Collection of Insects, Arachnids, and Nematodes. To clarify the identity of specimens belonging to the *Bracon* genus, all 16 of the individuals collected were sequenced. Genomic DNA was non-destructively extracted from the whole body of adult parasitoids, using a Chelex DNA extraction protocol (Walsh *et al*. 1991) that has been optimised for extraction of parasitoid DNA in 96-well plate format (T.D.G., unpublished).

A portion of the cytochrome *c* oxidase subunit 1 (COI) mitochondrial gene was amplified by polymerase chain reaction using universal primers LCO and HCO2198 (Folmer *et al*. 1994). Purified polymerase chain reaction products were sequenced bi-directionally (with above primers) on an ABI 3730 DNA analyser at the Robarts Research Institute (London Regional Genomics Centre, London, Ontario, Canada). Forward and reverse sequences were edited and assembled in Sequencher version 5.4.1 (available from www.genecodes.com) and were deposited in BOLD (www.boldsystems.org) and GenBank (www.ncbi.nlm.nih.gov/genbank/) databases. Interspecific distances were calculated using the K2P method in BOLD (Kimura 1980).

Results

Parasitoid emergence

A total of 2656 weevils were collected from 2604 aborted peppers at the 10 sample locations, averaging 1.02 weevils per pepper (Table 1). Samples collected from 16 September to 1 December 2016 were evaluated for pepper weevil and parasitoid emergence from aborted peppers (Fig. 1). Only one greenhouse site yielded no weevils. However, this may be due to an unfavourable pepper type or perhaps a low sample size being collected from this site. While most pepper weevil parasitoids were collected from one sample site in Cottam, Ontario, some specimens were collected from sample sites at least 14.8 km (*Nealiolus* Mason species 1), and 77.1 km (*Bracon* species) away from this location.

Based on morphology and genetic analysis, at least seven parasitoid species belonging to five genera were associated with pepper weevil (Table 2). *Jaliscoa hunteri*, a parasitoid previously reported from the pepper weevil in its native range, is reported from Canada for the first time, from a single sampling date at the organic greenhouse pepper site. *Pteromalus anthonomi*, a generalist known to be associated with a number of weevil species, has been previously reported from *Anthonomus signatus* Say (Coleoptera: Curculionidae) in Ontario (Yu 2018). A single *Eupelmus pulchriceps* Cameron (Hymenoptera: Eupelmidae), a known parasitoid of the pepper weevil and hyperparasitoid of parasitic wasps was also collected. At least four parasitoid species were identified as Braconidae, and included representatives from two subfamilies: members of

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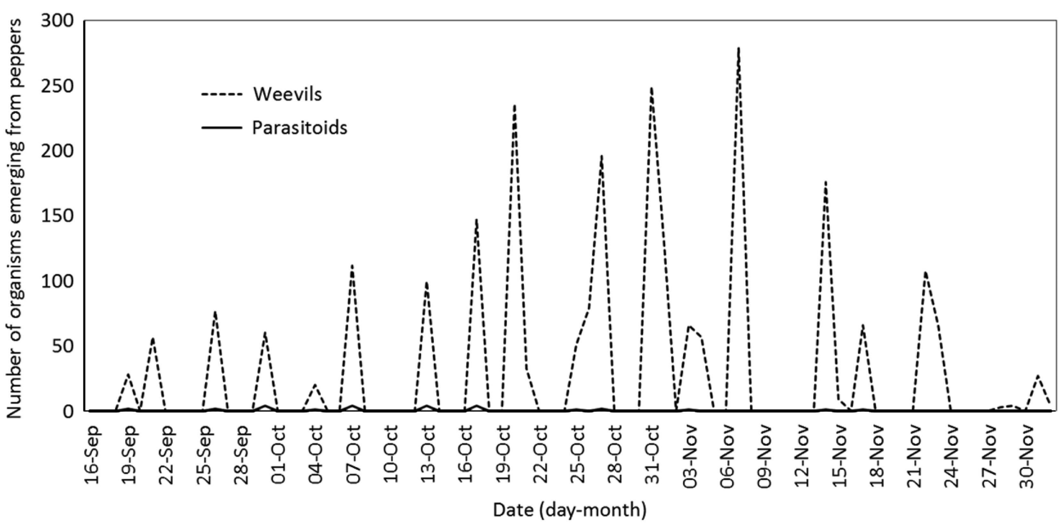
Table 1. Results of a 2016 survey of *Anthonomus eugenii* infested peppers from 10 locations in southwestern Ontario.

Sample site	Latitude	Longitude	Pepper types	Number of peppers collected ($n = 2604$)	Number of emergent weevils ($n = 2656$)	Number of emergent parasitoids ($n = 59$)	Rate of parasitism (%)
Kingsville 1*	42.05	−82.69	Jalapeno, Chili	222	204	1	0.49
Kingsville 2*			Scotch bonnet, Jalapeno	447	778	3	0.39
Kingsville 3*	42.03	−82.69	Hot cherry, Banana, Jalapeno	281	102	2	1.94
Grande Pointe‡	42.41	−82.36	Bell	28	11	0	0.00
Leamington 1‡	43.66	−79.41	Mini sweet	55	0	0	0.00
Leamington 2§	42.1	−82.59	Bell	200	55	6	10.71
Dresden*	42.48	−82.19	Banana	154	158	2	1.26
Wallaceburg*	42.56	−82.38	Hot cherry	10	5	0	0.00
Cottam†	42.14	−82.76	Hot cherry, Mini sweet, Bell, Jalapeno, Chili	1156	1246	45	3.61
Chatham*	42.44	−82.2	Jalapeno	51	97	0	0

Note: Parasitism = # parasitoids emerged/(# parasitoids + # pepper weevils) × 100 for each sample site.
* Traditional field practice.
† Organic field practice.
‡ Traditional greenhouse practice.
§ Organic greenhouse practice.

Fig. 1. Numbers of adult *Anthonomus eugenii* and hymenopteran parasitoids emerging from aborted peppers ($n = 2604$) of six different varieties over time in 2016.



the genus *Bracon* (Braconinae) and the genus *Nealiolus* (Brachistinae). Within *Nealiolus*, three species were confirmed based on distinct morphological and molecular differences observed between specimens. Of the 16 *Bracon* specimens collected and sequenced in this study, all appear to belong to the same species as they were found to have 1.37% or less sequence variation. However,

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Table 2. Parasitoids associated with *Anthonomus eugenii* from five southwestern Ontario sample locations.

Species	Family, subfamily	Number of parasitoids collected ($n = 43$)	Sample sites	Distance (km) from Cottam
Bracon species	Braconidae: Braconinae	6 ♀ 10 ♂	Cottam	NA
			Kingsville 1	14.4
			Chatham	77.1
Nealiolus species 1	Braconidae: Brachistinae	4 ♀ 2 ♂	Cottam	NA
			Kingsville 1	14.4
Nealiolus species 2	Braconidae: Brachistinae	2 ♀ 1 ♂	Cottam	NA
Nealiolus species 3	Braconidae: Brachistinae	1 ♀	Cottam	NA
Pteromalus anthonomi	Pteromalidae: Pteromalinae	8 ♀ 2 ♂	Cottam	NA
Jaliscoa hunteri	Pteromalidae: Pteromalinae	6 ♀	Leamington 2	17.2
Eupelmus pulchriceps	Eupelmidae: Eupelminae	1 ♀	Leamington 2	17.2

Note: The three *Nealiolus* species are distinguished as separate on the basis of distinct morphological and molecular characters.

some morphological variation does exist among these individuals so further examination of additional specimens and a more detailed investigation of their natural history and life cycle may be required to clarify the implications for this variation. In addition to the parasitoids listed above, two female parasitoids of a species of *Aphaereta* Förster (Hymenoptera: Braconidae) and six individuals of a species of *Asobara* Förster (Hymenoptera: Braconidae) were also recorded emerging from peppers. The latter two species are likely associated with flies such as *Scaptomyza adusta* (Loew) (Diptera: Drosophilidae), which also emerged from some infested peppers. This is also supported by the many records of these species parasitising dipteran hosts and the absence of evidence for their parasitism of coleopteran species (Yu 2018).

Genetic characterisation

Representative DNA sequences were obtained from the seven different parasitoid wasp species identified in this study. These have been deposited into Genbank and BOLD databases and include sequences from: *Bracon* species (658 base pairs) (BOLD:ADL4705), *J. hunteri* (652 base pairs) (BOLD:ADS6957), *Nealiolus* species 1 (658 base pairs) (BOLD:ACE7371), *Nealiolus* species 2 (658 base pairs) (BOLD:ACE7370), *Nealiolus* species 3 (658 base pairs) (BOLD:ADR5301), *P. anthonomi* (652 base pairs) (BOLD: AAU9445), and *E. pulchriceps* (658 base pairs) (BOLD:ACQ1025). Specimen information, sequences, and trace files are also available on BOLD (title: Project PEW Parasitoids of *Anthonomus eugenii*). While these preliminary DNA barcode data did not permit identification beyond what the morphological identifications provided, they did in some instances support the identity of specimens. For instance, the *Bracon* species COI sequence obtained in this study shared 89.97% similarity to that of a *Bracon* species voucher (BOLD:AAQ2969). Among the three *Nealiolus* voucher specimen barcodes, species 1 and 2 were 97.84% similar to each other, whereas species 2 and 3 were only 87.86% similar. In addition, when compared with their top matching sequences within the BOLD database, *Nealiolus* species 1 shared 98.46% similarity with a *Nealiolus* labelled specimen (BOLD: ACE7370), *Nealiolus* species 2 shared 99.07% similarity with the same *Nealiolus* labelled specimen (BOLD:ACE7370), and *Nealiolus* 3 shared 87.86% similarity with *Nealiolus* labelled specimen (BOLD:ACE7370). The *P. anthonomi* COI sequence obtained here shared 99.52% similarity to the COI sequence of a voucher specimen identified only to the Pteromalidae family level (BOLD:AAU9445). Records indicate that this specimen was collected from Algonquin Provincial Park, Ontario, Canada. The *J. hunteri* COI barcode sequence shared only 92.23% similarity to a voucher sequence labelled only to the Pteromalidae family level (BOLD:ABW8261). The *E. pulchriceps* COI sequence shared 98.33% similarity to that of voucher specimen sequence labelled *E. cushmani* (BOLD:ACQ1025). Together, the preliminary DNA barcode data

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generated here contributes to populating a DNA barcode database for parasitoids associated with the invasive pepper weevil. Once complete, this database will serve to assist with future identification and species separation efforts.

Discussion

The outbreak of the pepper weevil in southern Ontario in 2016 provided a timely and unprecedented opportunity to conduct a preliminary survey for natural enemies that can exploit this exotic pest as a host resource. These results are considered significant given the importance of this pest to the cultivation of pepper crops in North America and elsewhere, and given the propensity for the pest to transiently establish in different locations worldwide. Despite the relatively short duration of the survey (one field season), a surprisingly diverse parasitoid community was found in association with the pepper weevil. This community is strikingly similar to those known to be associated with the pepper weevil or other weevil species in Ontario and elsewhere.

There is a strong possibility that the parasitoid community associated to *A. eugenii* in Canada is the result of their prior association with a native weevil such as with the strawberry clipper weevil, *A. signatus*, an important pest of strawberries in Ontario (Ontario Ministry of Agriculture, Food, and Rural Affairs 2017). Like the pepper weevil, it is exploited by a diverse community of hymenopteran parasitoids, including *Bracon mellitor* Say, *Aliolus canadensis* (Provancher) (formerly *Eubazus canadensis*) (Provancher 1883), and *Nealiollus curculionis* (Fitch) (Braconidae); *Lariophagus fragariae* (Rohwer) (formerly *Catolaccus fragariae*) (Gibson 2013), *Pteromalus obscuripes* (Ashmead), *P. anthonomi*, and *J. hunteri* (Pteromalidae); and *Eupelmus vesicularis* (Retzius) (Eupelmidae) (Yu 2018). In the case of the unidentified *Nealiolus* species recovered from pepper weevil-infested peppers in this study, it is possible that further morphological and molecular analyses will demonstrate a close relation to *N. curculionis*, which may attack any *Anthonomus* species as available. It is also possible that the unidentified *Bracon* species discovered here could be *B. mellitor* Say (Hymenoptera: Braconidae), which is considered the most important parasitoid of the boll weevil, *Anthonomus grandis* Boheman (Coleoptera: Curculionidae) in the southeastern United States of America (Hunter and Hinds 1905; Pierce 1908), and is recognised as a parasitoid of pepper weevil elsewhere (Cross and Chesnut 1971). A survey of natural enemies of clipper weevils and other related weevils in Canada would help clarify these potential interactions.

The parasitoids associated with pepper weevil in this study also overlap considerably with species found associated with the pepper weevil in Mexico including *Bracon* species, *Aliolus* species, *J. hunteri*, and *Eupelmus* species (Rodríguez-Leyva *et al.* 2007). Findings from such studies may thus provide guidance for determining the most effective species for a biological control programme in Ontario. For example, in Mexico, *Bracon* species was the least abundant parasitoid and therefore unlikely to be effective as a biological control agent. In contrast, *J. hunteri* extensively parasitised the pepper weevil in Mexico, and is the primary parasitoid of pepper weevil in fields in Florida (Wilson 1986). In this study, we provide the first record for the occurrence of this species in Canada. While it is possible that this species is adventive, having been imported along with weevil-infested peppers from Mexico or Florida (Aguilar and Servín 2000; Rodríguez-Leyva *et al.* 2000), it may also have come into Canada from more proximal sources. For instance, *J. hunteri* has been previously recorded in Washtenaw County, Michigan, United States of America (Gibson 2013), which is ~120 km away from the site at which the species was recorded in Canada.

Missing from this initial survey of the parasitoid complex in Ontario is *T. eugenii* Wharton and Lopez-Martinez (Hymenoptera: Braconidae) another important parasitoid species attacking this pest in Mexico (Wharton and López-Martínez 2000). As it is currently known to be associated with only two weevil species, *A. eugenii* and *A. sisyphus* Clark (Coleoptera: Curculionidae) (Yu 2018), *T. eugenii* would be a good initial candidate for evaluation as biological control agent of pepper weevil. Similarly, *P. anthonomi* also seems to have a relatively narrow host range, associated with only four *Anthonomus* species as well as *Lixus musculus* Say (Coleoptera: Curculionidae) (Yu 2018). The narrower host range of these two species make them safer options as candidate biological control

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agents relative to *Nealiolus* species and *J. hunteri*, which have broader host ranges. Furthermore, *E. pulchriceps* is known to act as a hyperparastoid with the potential to attack primary pepper weevil parasitoids (Gibson 2011). It may therefore actually reduce the efficacy of other parasitoid agents employed in biological control.

Conclusion

The findings of this study serve as a preliminary basis to elucidate the associations between locally occurring parasitoids that may successfully exploit *A. eugenii* as it potentially expands into a new geographic range, either as a transient or permanent population. This first step provides evidence that natural enemies are capable of exploiting the pepper weevil in southern Ontario. Subsequent steps should include additional surveys that cover a broader geographic range, and evaluation of the parasitoid community over successive field seasons to determine the prevalence of the different species and exploitation of alternative hosts. In addition, further genetic characterisation of these parasitoid communities will permit for more accurate assignments to taxonomic groups and help clarify host-parasitoid associations. While there are currently insufficient barcodes available in public databases to characterize the genetic variation within and among species, this and other initiatives will serve to eventually address this lack. Collectively, these steps are essential to determine which parasitoid species are capable of effectively suppressing populations of the pepper weevil or whether they are generalist parasitoids that are exploiting a transient resource at a low level. Should one or more of these species provide promise as a candidate biological control agent, further exploration of host-parasitoid associations and host specificity will be the next logical step in the potential development of an augmentative or inundative biological control programme targeting the pepper weevil in field and greenhouse pepper crops.

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