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Authors: Jessica Awad, Amanda Hodges, Stephen Hight, Mrittunjai Srivastava, Amy Howe, et. al.

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Laboratory rearing and sex ratio of *Apanteles opuntiarum* (Hymenoptera: Braconidae), a potential biocontrol agent of *Cactoblastis cactorum* (Lepidoptera: Pyralidae)

Jessica Awad^{1,2,*}, Amanda Hodges², Stephen Hight³, Mrityunjai Srivastava¹, Amy Howe¹, and Eric Rohrig¹

Abstract

The cactus moth, *Cactoblastis cactorum* (Berg) (Lepidoptera: Pyralidae), is an invasive species in North America, where it poses a threat to species of *Opuntia* Mill. of economic and ecological importance. The parasitoid *Apanteles opuntiarum* Martinez & Berta (Hymenoptera: Braconidae) is currently under evaluation as a potential biological control agent. This study was conducted to develop a parasitoid rearing protocol, with special attention to laboratory sex ratio and the effects of inbreeding. The parasitoid rearing method used a natural cactus host diet for culture of the moths. Female wasps were mated with siblings, non-siblings, or a combination. Clutch size, clutch number, and offspring sex ratios were recorded. The effects of sibling mating on these factors were analyzed. Offspring of sibling-mated parasitoids exhibited a significant increase in female sex ratio. The rearing method produced 6 successive generations in captivity with no additional introductions of genetic material. Hence, the protocol appears suitable for long-term maintenance of quarantine colonies. The effects of inbreeding suggest that natural populations of *A. opuntiarum* are subject to local mate competition. Therefore, some amount of inbreeding is recommended for maintenance of an optimal sex ratio of *A. opuntiarum* in laboratory colonies.

Key Words: local mate competition; inbreeding; *Opuntia*; invasive species

Resumen

La polilla del cactus, *Cactoblastis cactorum* (Berg) (Lepidoptera: Pyralidae), es una especie invasora en América del Norte, donde representa una amenaza para las especies de importancia económica y ecológica de *Opuntia* Mill. El parasitoide *Apanteles opuntiarum* Martinez & Berta (Hymenoptera: Braconidae) está actualmente bajo evaluación como un agente potencial de control biológico. Este se realizó estudio para desarrollar un protocolo de cría para el parasitoide, con atención especial a la proporción de hembras a machos en el laboratorio y los efectos de la endogamia. El método de crianza del parasitoide utiliza una dieta de cactus para criar las polillas. Las avispas hembras se aparearon con sus hermanos, con otros que no fueron hermanos o una combinación de los dos. Se registraron el tamaño del nido de huevos, el número de huevos en el nido y la proporción de hembras a machos de la descendencia. Se analizaron los efectos del apareamiento entre hermanos en estos factores. La descendencia de parasitoides apareados con sus hermanos mostró un aumento significativo en la proporción de hembras. El método de crianza produjo 6 generaciones sucesivas en cautiverio sin introducciones adicionales de material genético. Por lo tanto, el protocolo parece adecuado para el mantenimiento a largo plazo de las colonias de cuarentena. Los efectos de la endogamia sugieren que las poblaciones naturales de *A. opuntiarum* están sujetas a la competencia local de parejas. Por lo tanto, se recomienda cierta cantidad de endogamia para mantener una proporción de sexos óptima de *A. opuntiarum* en colonias de laboratorio.

Palabras Clave: competencia de pareja local; endogamia; *Opuntia*; especies invasivas

The invasive cactus moth, *Cactoblastis cactorum* (Berg) (Lepidoptera: Pyralidae) is native to Argentina, Uruguay, Paraguay, and southern Brazil. Its larvae are oligophagous on most species of *Opuntia* Mill. (Caryophyllales: Cactaceae) (Mann 1969). Although originally introduced to the Caribbean as a biological control agent for indigenous *Opuntia* spp., *C. cactorum* became an invasive species in the region (Zimmermann et al. 2001). An established population was found in the Florida Keys in 1989, and by 2009 it had spread as far north as North Carolina and as far west as Louisiana (Dickel 1991; Hight & Carpenter 2009). *Cactoblastis cactorum* poses a threat to ecologically important and endangered cactus species in Florida, and has the potential to be-

come a serious economic pest in Texas and Mexico, where *Opuntia* species are used for forage, food, fuel, and dye production (Zimmermann et al. 2004).

Chemical control of *C. cactorum* is not recommended due to its presence in ecologically sensitive natural areas (Pemberton & Cordo 2001). Eradication via sanitation, pheromone trapping, and sterile insect technique is effective over small areas, but the current range of *C. cactorum* is too large for this to be economically feasible (Varone et al. 2015). Classical biological control can provide a sustainable method of suppressing widespread populations of invasive species. However, imported biological control agents must be assessed for host specific-

¹Florida Department of Agriculture and Consumer Services, Division of Plant Industry, Gainesville, Florida 32608, USA; Email: jessica.awad@freshfromflorida.com (J. A.); mritunjai.srivastava@freshfromflorida.com (M. S.); amy.howe@freshfromflorida.com (A. H.); eric.rohrig@freshfromflorida.com (E. R.)

²University of Florida, Department of Entomology and Nematology, Gainesville, Florida 32611, USA; Email: achodges@ufl.edu (A. H.)

³USDA, Agricultural Research Service, Tallahassee, Florida 32308, USA; Email: stephen.hight@ars.usda.gov (S. H.)

*Corresponding author; Email: jessica.awad@freshfromflorida.com

ity to prevent undesirable non-target effects (Strand & Obrycki 1996). *Apanteles opuntiarum* Martinez & Berta (Hymenoptera: Braconidae) is a gregarious larval endoparasitoid of *C. cactorum* and *Cactoblastis doddii* Heinrich (Lepidoptera: Pyralidae) in Argentina (Martinez et al. 2012; Varone et al. 2015). This narrow host range makes it a promising candidate for biological control of *C. cactorum* in North America.

A successful parasitoid rearing program serves to ensure the identity of the beneficial organisms; to determine their reproductive ability in the laboratory; and to allow the conduct of research on their biology, including host specificity, to determine suitability for release (Ertle 1993). The development of a laboratory rearing protocol for *A. opuntiarum* will allow future workers to obtain consistent reproduction, optimal parasitism rate, and sufficient numbers to continue with the biological control evaluation process. Information related to optimal sex ratio is important for the culture of successive generations in the laboratory. Hymenoptera have haplodiploid sex determination, meaning that unfertilized eggs become males (haploid) and fertilized eggs become females (diploid). Female parasitoids may adjust offspring sex ratio in response to environmental conditions, host quality, and inbreeding status (Godfray 1994).

The effects of inbreeding are particularly important to quarantine colonies, as obtaining new genetic material from outside the country may be expensive or otherwise difficult. The natural mating structure of a species, especially the frequency of local mating, can influence its response to inbreeding in the laboratory (Hardy 1994). The theory of local mate competition predicts a positive correlation between inbreeding and proportion of female offspring (Hamilton 1967). Local mate competition tends to occur in gregarious species whose hosts are patchy in distribution. In these species, male siblings often compete to mate with their sisters immediately after emerging. Therefore, production of male offspring should be lower under high-inbreeding conditions (Werren 1987; Hardy 1994). Little is known of the natural mating structure of *A. opuntiarum*, but it is a gregarious parasitoid, males emerge before females, and hosts may be patchily distributed (Varone et al. 2015). If local mate competition applies to *A. opuntiarum*, inbreeding in captivity theoretically should promote a high proportion of females in the colony.

The primary goal of this study was to describe a laboratory rearing method for *A. opuntiarum*, with special attention to sex ratio and the effects of inbreeding. We hypothesized that sibling mating of *A. opuntiarum* produces higher proportions of female offspring than non-sibling, outcrossed mating.

Materials and Methods

REARING CONDITIONS

Experiments were conducted at the facilities of the Florida Department of Agriculture and Consumer Services, Division of Plant Industry in Gainesville, Florida, USA. *Cactoblastis cactorum* larvae were mass reared on an artificial diet (Marti et al. 2008). *Apanteles opuntiarum* were reared under quarantine conditions. Temperatures in the containment facility ranged between 20 and 25 °C, and relative humidity ranged between 50% and 70%. All treatments experienced a 16:8 h (L:D) photoperiod.

APANTELES OPUNTIARUM REARING

The *A. opuntiarum* rearing method described below was developed in 2015 as a component of this project. Host-parasitoid ratios and numbers follow Mengoni Gonalons et al. (2014). Parasitized *C. cac-*

torum larvae were collected in Dec 2015 and Mar 2016 from field sites in Cordoba, Catamarca, and Santiago del Estero, Argentina. Laboratory populations of *A. opuntiarum* originated from this material.

Although *C. cactorum* can be reared efficiently on artificial diet, hosts fed with *Opuntia* spp. cladodes were significantly more attractive to female parasitoids (Varone et al. 2016,2017). To serve as optimal hosts for *A. opuntiarum*, 35 *C. cactorum* larvae (second to third instar) were transferred from artificial diet blocks to a slice of *Opuntia* cladode (20 × 7 cm) and allowed to feed for 24 h before exposure to the parasitoid.

Six adult *A. opuntiarum* (2 females and 4 males) were placed in a 1.9 L glass canning jar with a modified 80-mesh lid (Fig. 1). A small slice of *Opuntia* (2 × 2 cm) and a damp strip of paper towel (1 × 3

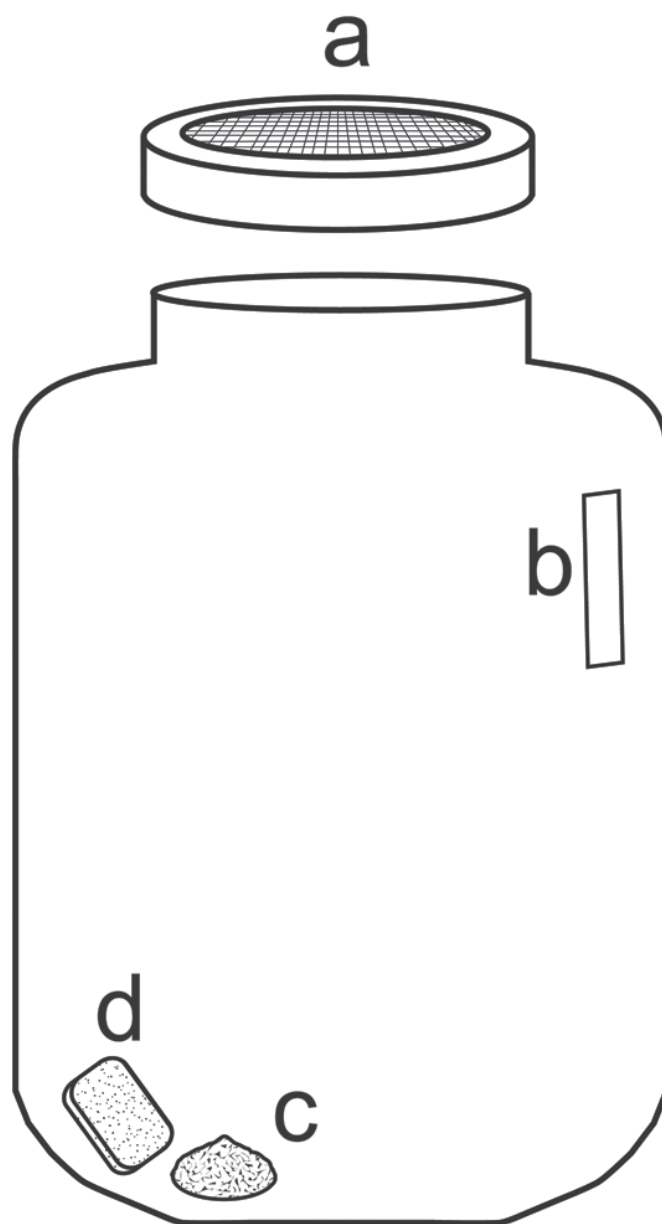


Fig. 1. Initial mating jar arrangement for *Apanteles opuntiarum*. A 1.9 L glass canning jar contained 2 adult female and 4 adult male *A. opuntiarum*. The wasps were left to mate for 24 h before the introduction of host material. (a) Mesh lid with honey drop. (b) Paper towel strip with water. (c) Frass of cactus-fed *Cactoblastis cactorum*. (d) Small slice of *Opuntia* tissue.

cm, Kimberly-Clark Wypall L30, Kimberly-Clark Corp., Roswell, Georgia, USA) were added to provide moisture. Honey was applied to the mesh lid with a paintbrush to provide an energy source. A dollop of frass from *C. cactorum* was applied to the bottom of the jar. To avoid oviposition before mating, *A. opuntiarum* males and females remained in the jar without *C. cactorum* for 24 h. After this 24 h mating period, the slice of *Opuntia* and 35 *C. cactorum* larvae were added to the jar (Fig. 2). *Apanteles opuntiarum* and *C. cactorum* remained together in the jar for 6 d.

After the 6 d exposure period, *C. cactorum* larvae and *Opuntia* were transferred to a plastic tub (19 × 29 × 9 cm) with a ventilated lid (2 circular holes, covered with 80 mesh, 6.5 cm diam). Each tub contained a wire grid (10 × 27 cm), where the long ends were bent at right angles to form a support structure for the *Opuntia* cladodes (Fig. 3). Approximately 150 mL of unscented cat litter (Special Kitty Unscented Natural Clay Cat Litter, WalMart Inc., Bentonville, Arkansas, USA) was added to each container to absorb frass and cactus fluids.

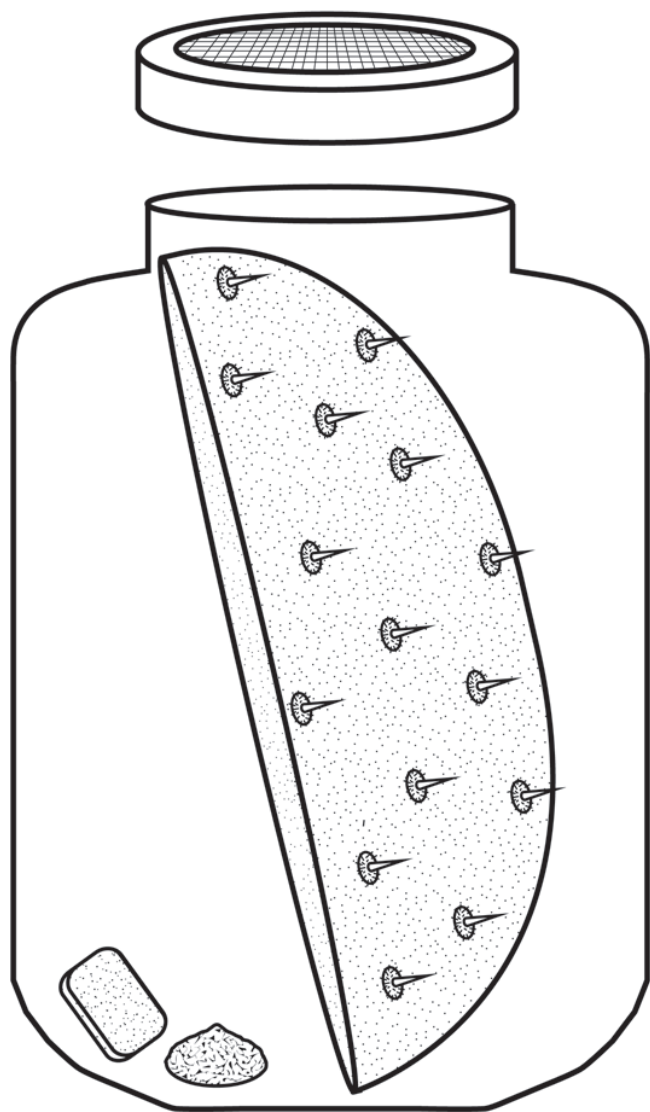


Fig. 2. Mating jar arrangement, 24 h after initial set-up. Host material was introduced in the form of an *Opuntia* cladode containing 35 *Cactoblastis cactorum* larvae (instars 2 and 3). The position and size of cladode are shown relative to the 1.9 L glass canning jar.

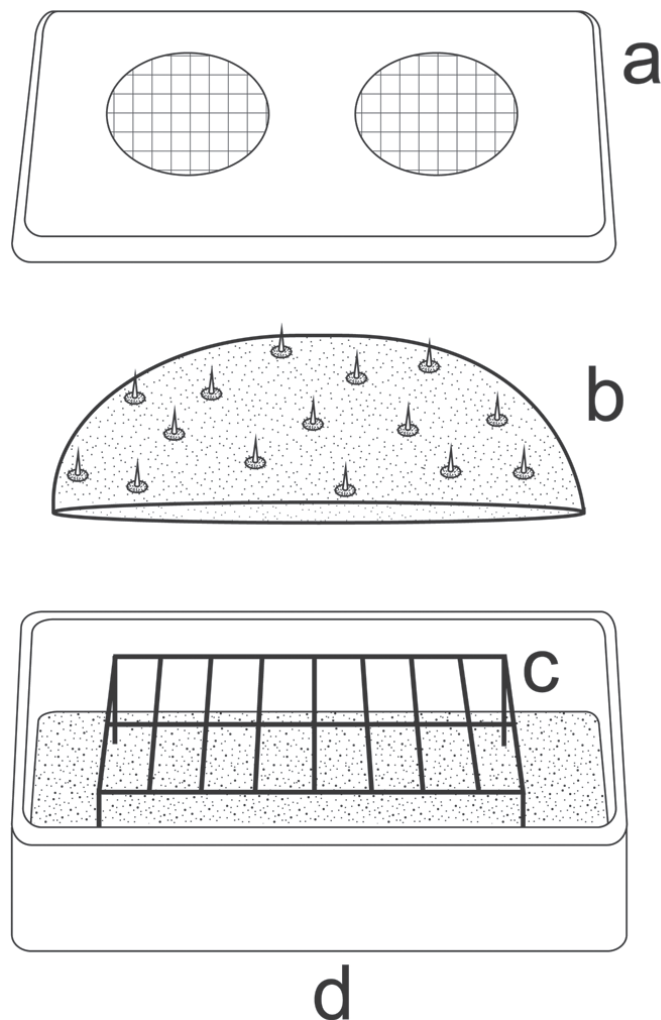


Fig. 3. Rearing container for *Cactoblastis cactorum* that were exposed to *Apanteles opuntiarum*. After the 6 d exposure period, *Opuntia* cladode and *C. cactorum* larvae were moved to this plastic container. (a) Plastic lid with mesh cut-outs. (b) *Opuntia* cladode containing 35 *C. cactorum* larvae. (c) Wire rack to elevate host material above substrate. (d) Plastic tub containing unscented cat litter.

Every 2 to 3 d, *C. cactorum* frass was removed, *Opuntia* tissue was replaced, clean litter was added, and pupae of *C. cactorum* and *A. opuntiarum* were counted and removed. Pupae of *C. cactorum* were discarded. Clutches of *A. opuntiarum* pupae that had erupted from their *C. cactorum* host larva were removed intact, and placed into plastic Petri dishes. Each Petri dish contained a drop of honey on the lid and a strip of damp paper towel. A small slice of *Opuntia* (2 × 2 cm) was added to each Petri dish to provide moisture over weekends and holidays.

Upon emergence, adult *A. opuntiarum* were counted, sexed, and individually separated into 15 mL plastic test tubes. A minute drop of honey was placed on each lid. A strip of damp paper towel was placed over the edge of each tube. When the paper became dry, water was added to the external edge of the paper, allowing the provision of moisture without opening the tube.

SIBLING MATING TESTS

To observe the effect of inbreeding on sex ratio, female *A. opuntiarum* from the same clutch were mated with brothers and non-

brothers in different jars. Due to variation in the number of pupae and sex ratio, sometimes the necessary numbers were not available, and inbreeding and crossbreeding experiments used females from different clutches rather than 2 pairs of sibling females. Also, on occasion we used a small number of jars that contained a combination of sibling and non-sibling *A. opuntiarum*, if they were the only adults available at the time. These data were analyzed separately.

In total, 193 jars contained unrelated males and females (crossbred treatment), 44 jars contained sibling males and females (inbred treatment), and 32 jars contained a mixture of siblings and non-siblings (mixed treatment). The uneven sample sizes were due to a limited number of sibling wasps. Because a wasp has a limited number of brothers and a virtually unlimited number of non-brothers, only 44 sibling-mated treatments could be created from the laboratory population over the course of the study. Some sibling mating occurred within Petri dishes before individual *A. opuntiarum* could be separated, so the rate of inbreeding is likely higher than indicated by the experimental design.

Clutch size (number of parasitoids per clutch), clutch number (number of clutches per jar), offspring sex ratio, and total numbers of male and female offspring were recorded from the laboratory colony of *A. opuntiarum*. Effects of sibling mating were analyzed by the Kruskal-Wallis rank sum test. For variables with a significant ($P \leq 0.05$) Kruskal-Wallis test, post-hoc analysis was performed using the pairwise Mann-Whitney U test. Kruskal-Wallis and Mann-Whitney analyses were conducted using R statistical software (R Core Team 2015).

Results

APANTELES OPUNTARUM REARING

Six generations of *A. opuntiarum* were obtained using this rearing method. The proportion of jars yielding parasitoid offspring was 59%, and the overall parasitism rate of all host larvae was 10%. In total, 158 of 269 mating jars produced parasitized *C. cactorum*, resulting in 602 clutches of *A. opuntiarum* (Table 1). Reproduction of *A. opuntiarum* occurred in 114 of the crossbred jars, 27 of the inbred jars, and 17 of the mixed jars. Successful production of female offspring occurred in 73 of the crossbred jars, 21 of the inbred jars, and 13 of the mixed jars. The inbred mating jars exhibited the highest percentage of offspring production and female offspring production.

SIBLING MATING TESTS

Kruskal-Wallis analysis of clutches by parental relatedness (Table 2) demonstrates a significant relationship between the proportion of female offspring and inbreeding status ($H = 13.925$; $df = 2$; $P < 0.001$). The mean female offspring per jar did not vary between treatments ($P > 0.4$), although the crossbreeding treatments produced noticeably higher (but statistically insignificant) numbers of male offspring ($P >$

0.2). Clutch size and clutch number did not differ significantly between treatments ($P > 0.05$).

Discussion

Our experiments demonstrated a significant relationship between sibling mating and offspring sex allocation in *A. opuntiarum*. Sibling-mated *A. opuntiarum* produced a higher proportion of female offspring per clutch ($n = 602$) than did those mated with non-siblings. When using the jar as the statistical unit, the results were not significant, possibly due to the low sample number ($n = 44$). However, the trend was consistent with the hypothesis of local mate competition (Hamilton 1967). We conclude that some degree of local mate competition occurs in natural populations of *A. opuntiarum*.

Different responses to sibling mating may reflect different natural mating structures, or may occur due to experimental conditions. Experimental inbreeding of other haplodiploid species shows a variety of effects (Table 3). In most of these experiments, male-biased sex ratios resulted from inbreeding treatments, or no effect was observed. Female offspring and inbreeding were correlated only in the eusocial ant *Cardiocondyla obscurior* Wheeler (Hymenoptera: Formicidae) (Schrempf et al. 2006). It is unclear why *C. obscurior* should be subject to local mate competition, but it may be linked to the capacity to adopt unrelated queens, or to male dispersal dimorphism (de Menten et al. 2005).

Among parasitoid species, experimental comparisons of inbred and outbred offspring sex ratios are not the only approach to evaluating the influence of local mate competition on sex allocation. Many studies have used a foundress number as a proxy for inbreeding status (Herre 1987; King 1987; Somjee et al. 2011). In the case of *Nasonia vitripennis* (Walker) (Hymenoptera: Pteromalidae), laboratory inbreeding does not affect offspring sex ratio, but the manipulation of foundress numbers supports the theory of local mate competition (Werren 1983). Therefore, lack of response to laboratory inbreeding does not necessarily mean that local mate competition has no impact. In the *A. opuntiarum* experiments, foundress numbers and relatedness were controlled (2 female siblings in each mating jar).

There are 2 possible inferences about the natural mating structure of *A. opuntiarum* based on laboratory sex ratios. First, sibling mating predominates in the field, and the high proportion of male offspring from crossbred pairs is a symptom of outbreeding depression. Second, natural populations are subject to a mixture of inbreeding and outbreeding. In this scenario, an adjustable sex ratio allows female wasps to maximize offspring relatedness according to the availability of sibling vs. non-sibling mates. In the second case, but not in the first, long-term inbreeding may exhibit deleterious effects on laboratory populations. Further observation of the captive colony at the Division of Plant Industry may shed light on this distinction.

Table 1. Production of *Apanteles opuntiarum* offspring from rearing jars treated with 3 different parental mating scenarios.

Treatment	Number of jars	Number of jars producing offspring	Percentage of jars producing offspring	Percentage of jars producing female offspring	Total number of clutches
Crossbred	193	114	59%	38%	446
Inbred	44	27	61%	48%	96
Mixed	32	17	53%	41%	60
All	269	158	59%	40%	602

Table 2. Mean (\pm SD) clutch size (number of parasitoids per clutch), number of clutches per jar, female sex ratio, and number of female or male *Apanteles opuntiarum* offspring produced from mating jars treated with 3 different parental mating scenarios.

Treatment	Clutch size	Clutch number per jar	Proportion female	Female offspring per jar	Male offspring per jar
Crossbred	16 \pm 11.9 a	4 \pm 4.4 a	0.41 \pm 0.39 a	22 \pm 40.8 a	38 \pm 63.9 a
Inbred	12 \pm 10.5 a	4 \pm 4.3 a	0.57 \pm 0.35 b	27 \pm 45.1 a	18 \pm 27.7 a
Mixed	13 \pm 8.7 a	4 \pm 1.7 a	0.47 \pm 0.37 ab	24 \pm 26.3 a	23 \pm 23.9 a

Values in a column followed by different lowercase letters are significantly different ($P \leq 0.05$; Kruskal-Wallis rank sum and Mann-Whitney U tests).

The effects of *Wolbachia* on sex ratios of *A. opuntiarum* remain unclear. Mengoni Gonalons et al. (2014) diagnosed *Wolbachia* infection in both captive and wild individuals. The strain belongs to supergroup B, and has a partial coincidence with a strain known from parthenogenetic South American weevils (Coleoptera: Curculionidae). The reproductive effects of *Wolbachia* include cytoplasmic incompatibility, which could inhibit fertilization of females by unrelated males (Werren et al. 2008). Experimental manipulation of *Wolbachia* infection via antibiotics was beyond the scope of this study, but could provide an interesting avenue for future research.

The rearing protocol was successful in producing multiple generations of parasitoids, with sufficient numbers of female offspring to maintain the colony and conduct host range testing. However, successful parasitism occurred in fewer than 62% of jars. Within these, the average parasitism rate was less than 11%. Overall, parasitism should be improved to increase rearing efficiency and reduce costs. Future work may concentrate upon manipulation of environmental variables to increase parasitism rate in captivity.

The *A. opuntiarum* rearing program represents the first successful application of the local mate competition theory to increase the proportion of female offspring via inbreeding of an ichneumonoid biological control agent. There remains much opportunity for the development and improvement of similar rearing protocols for other parasitoid species of economic importance. Future attempts to manipulate

parasitoid sex ratio via sibling mating should focus upon gregarious hymenopteran species with early male emergence.

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Table 3. Review of various studies measuring the effect of experimental inbreeding on sex ratio in haplodiploid Hymenoptera.

Family	Genus and species	Proportion male (M) or female (F)	Inbreeding	Effect of inbreeding	Reference
Braconidae	<i>Cotesia glomerata</i>	0.56 M	Y	more males	Gu & Dorn 2003
		0.39 M	N		
Braconidae	<i>Aphidius rhopalosiphii</i>	0.68 M	Y	more males	Salin et al. 2004
		0.38 M	N		
Braconidae	<i>Cotesia glomerata</i>	0.435 M	Y (F1)	more males	Zhou et al. 2007
		0.558 M	Y (F2)		
		0.664 M	Y (F3)		
		0.7 M	Y (F4)		
		0.219 M	N		
Ichneumonidae	<i>Mastrus ridens</i>	0.74 M	Y	more males	Zaviezo et al. 2018
		0.64 M	N		
Figitidae	<i>Leptopilina heterotoma</i>	0.65 M	Y	complex	Hey & Gargiulo 1985
		0.68 M	N		
Pteromalidae	<i>Nasonia vitripennis</i>	0.4 M	Y	none	Reece et al. 2004
		0.4 M	N		
		0.25 M	Y		
		0.25 M	N		
Pteromalidae	<i>Nasonia vitripennis</i>	0.3 M	Y	none	Shuker et al. 2004
		0.3 M	N		
		0.4 M	Y		
		0.4 M	N		
Trichogrammatidae	<i>Trichogramma nr. brassicae</i>	0.63 F	Y	none	Sorati et al. 1996
		0.63 F	N		
Formicidae	<i>Cardiocondyla obscurior</i>	0.58 F	Y	more females	Schrempf et al. 2006
		0.35 F	N		

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