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Source: Journal of Economic Entomology, 115(4) : 1170-1176

Published By: Entomological Society of America

URL: https://doi.org/10.1093/jee/toac082

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Journal of Economic Entomology, 115(4), 2022, 1170–1176
https://doi.org/10.1093/jee/toac082
Advance Access Publication Date: 28 May 2022
Research

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Field and Forage Crops

Examining Historical Rates of Leafcutting Bee Brood Cell Pathogens, Parasitoids, and Predators to Establish Baseline Infectivity Rates for Alfalfa Seed Growers

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Subject Editor: Sally Taylor

Received 2 March 2022; Editorial decision 28 April 2022.

Abstract

The alfalfa leafcutting bee *Megachile rotundata* (Fabricius) (Hymenoptera: Megachilidae) is the primary pollinator for the alfalfa seed industry. It is a solitary cavity nesting bee that utilizes leaf lined brood cells provisioned with pollen for larval development and pupation into the adult stage. During development, multiple pathogens, parasitoids, and predators can prey upon or use the larvae as a host, resulting in the loss of the future adult bee. As such, the presence of invertebrate pests and fungal pathogens presents a major concern for commercial alfalfa seed growers. In the present study, we used historic data from the Parma Cocoon Diagnostic Laboratory to determine baseline rates of pathogens, parasitoids, and predators of *Megachile rotundata* brood cells and used this analysis to determine cutoffs for management practices to inform growers when the purchase of new bee stocks should be considered. Additionally, we compared the presence of chalkbrood, predators, and parasitoids in samples collected from both grower-produced stocks and newly purchased Canadian bees. The results of the investigation provide historic averages of the presence of chalkbrood, predators, and parasitoids, show a significant increase in chalkbrood and predators in 2007–2011, and find a significant difference in rates of chalkbrood and predators between samples from Canadian suppliers and grower stocks. We speculate that these differences may have resulted from economic conditions that increased the cost of Canadian *Megachile rotundata* cells and likely resulted in increased reliance on 2nd-year U.S. grower stocks and subsequently increased infection rates during this time period.

Key words: *Megachile rotundata*, Historical data analysis, chalkbrood, X-ray

Alfalfa (*Medicago sativa* L.) is an important agricultural commodity with high production value throughout the United States (Pitts-Singer and James 2008, NAAIC 2017, USDA NASS 2018). Alfalfa, excluding other hay production, encompasses approximately 6.8 million ha of arable land within the United States, with a production value of over 8.8 billion dollars annually (NAAIC 2017, USDA NASS 2018), and alfalfa production provides a vital resource for the livestock industry in the form of feed (hay and silage) (Conrad and Klopfenstein 1988). Continued success of alfalfa production requires a supply of healthy pollinators adequate to sustain alfalfa seed crops (Barnes 1980). Alfalfa seed production provides the germplasm to be used in the production of alfalfa hay. One of the primary seed producing regions in the world is the Pacific Northwest of the United States (Idaho, Oregon, and Washington; Mueller 2008). This agriculturally intense alfalfa seed production area is located southwest Idaho, southeast Oregon, and southcentral Washington.

Alfalfa is partially self-incompatible, and an insect pollinator is considered necessary to generate a seed crop. As such, alfalfa seed producers rely heavily on pollination from the alfalfa leafcutting bee, *Megachile rotundata* (Fabricius) (Hymenoptera: Megachilidae), and on the ground dwelling alkali bee (*Nomia melanderi*) in the Walla Walla Valley in Washington, as these bee species are able to

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trip the sexual column of the alfalfa flower at a significantly higher rate when compared with other pollinators including *Apis mellifera* ((Bohart 1962, Cane 2002, Cane 2008, Pitts-Singer and Cane 2011). *Megachile rotundata* is a solitary, gregarious cavity-nesting bee that does not form a beehive or produce honey. Instead, a single female *M. rotundata* will construct a nest that consists of individual brood cells that are lined with cut leaves (Pitts-Singer and Cane 2011, MacIvor 2016). Female bees will gather nectar and pollen and place them within the constructed nest cell (Pitts-Singer 2004, Cane et al. 2011, Pitts-Singer and Cane 2011). The female bee will lay a single egg within the constructed cell and seal in the egg (at this point the nest cell can be considered a brood cell) to undergo larval development and pupation into the adult stage (Pitts-Singer and Cane 2011). The production of the nest cell and resources needed to provide nutrition to the developing larvae requires multiple trips to flowering plants, resulting in the highly desirable trait of a very effective pollinator species (Pitts-Singer and James 2008, Pitts-Singer and Cane 2011).

Megachile rotundata population stocks can either be purchased from a commercial vendor in the United States or Canada, or propagated by growers in bee boards (above ground artificial structures that provide cavities for nesting bees) within their own fields over multiple years (Pitts-Singer and Cane 2011). One of the primary *M. rotundata* producing areas that growers purchase bee stocks from is in the central provinces of Canada, where *M. rotundata* are commercially propagated and used to pollinate canola and alfalfa seed (Richards 1984, Pitts-Singer and Cane 2011). In these high latitudes with short growing seasons, *M. rotundata* will produce one brood per season. In contrast, grower produced bees in the United States may have two generations per year under certain conditions. Historically, *M. rotundata* were produced in break-apart wooden bee boards from which the brood cells were removed and sold as loose cells. Under current practices, commercially managed *M. rotundata* are instead provided with fabricated polystyrene foam bee boards to nest in. Foam bee boards are removed from the agricultural fields and transported to controlled cold rooms for storage. Canadian brood cells are sold to U.S. alfalfa seed producers as first year stock (Pitts-Singer and Cane 2011). *Megachile rotundata* are generally sold as units described as gallon of bees which encompasses approximately 10,000 live larvae. These can be purchased in the polystyrene boards or as loose cells punched out of the boards (Pitts-Singer 2013). Loose cells can be sorted and cleaned to removed damaged or poor-quality cells using gravity tables or a tumbling system before the cells are placed in cold storage. This has been demonstrated to reduce the proportion of pollen balls cells in processed samples (James and Pitts-Singer 2013). Sex ratio is male biased and varies from 30% to 40% female (Pitts-Singer and James 2005). Typical release rates by growers in the United States are between 10 and 15 gallons per hectare (Pitts-Singer and Cane 2011). It is generally considered that the Canadian bee stocks have a lower presence of invertebrate pests and fungal pathogens (e.g., chalkbrood) due to their management practices and the use of paraformaldehyde to kill fungal spores (Pitts-Singer and Cane 2011). Supply and demand and currency exchange rates between the United States and the Canadian dollar dictate the price paid for *M. rotundata* by U.S. alfalfa seed growers, and the health of *M. rotundata* bee broods are a large concern for U.S. alfalfa seed growers since the purchase of *M. rotundata* can account for 20 to 40% of the operating expenses.

A major concern for commercial alfalfa seed growers is the presence of dead larvae, pollen ball (no visible larvae), invertebrate pests, and fungal pathogens within their bee stocks. During larval development within brood cells of *M. rotundata,* multiple pathogens and parasitoids can attack and prey on the developing larvae, resulting in the loss of the future adult bee, and therefore reducing future pollination services. Fungal pathogens include multiple *Ascosphaera* species that result in the disease phenotype known as chalkbrood due to the fungal sporulation that causes bee larvae to become hard and chalky (James and Pitts-Singer 2008). Alfalfa seed growers are predominately concerned with the presence of *Ascosphaera aggregata* within cells, as it is currently thought to be the predominant *Ascosphaera* species that results in *M. rotundata* cell loss (Goerzen 2002). Besides fungal pathogens, brood cells are also predated on and killed by multiple different parasitic wasp species including *Monodontomerus obscurus*, *Leucospis affinis*, *Pteromalus venustus*, and *Sapyga pumila* (Eves et al. 1980), nest destroying beetles including *Tribolium audax*, *Tribolium brevicornis*, *Trichodes ornatus* (Eves et al. 1980), and cuckoo bees (*Coelioxys rufocaudata*) (Eves et al. 1980). The presence of these pathogens, predators and parasitic wasps results in the reduction of growers' bee stocks (Brindley 1976, Stephen and Undurraga 1976, Pitts-Singer and James 2008). Additionally, these pathogens and predators can reproduce within grower stocks, bee boards, and housing, and if not controlled, can result in high abundance of dead bee larvae. Traditionally, growers monitor the presence of chalkbrood, parasitic wasps, and predators using a service that employs X-ray imaging as a diagnostic technique tool (Stephen and Undurraga 1976). The concern over the presence of predators, parasites, and pathogens has resulted in regulations, including statutes in Wyoming to mitigate the movement of any pest effecting *M. rotundata.* In order to reduce cell loss, growers can use a combination of disinfectants and lures to protect bee cells from different pathogens and parasitoids. However, if stocks contain a high percent of any of these pests, growers are forced to burn/bury their current bee cell stocks, sterilize bee boards and housing, and purchase new bee cell stocks from commercial vendors (Davis et al. 1979, Hill et al. 1984, James 2011). Currently, the acceptable cutoffs for any of these predators, pathogens, and parasitoids are not well defined.

In the current investigation, we examined archived data collected from the Parma Cocoon Diagnostic Laboratory (PDCL) from 1997 to 2021 to examine and reveal historical trends in the presence of pathogens and parasitoids infesting *M. rotundata* cells. These records provide baseline yearly infection rates of chalkbrood, predators, and parasites within historical samples and provide insights for growers regarding expectations and cutoffs for future *M. rotundata* stocks. We also examined the sex ratio of emerged *M. rotundata* adult bees to gain insight into the relative number of female bees emerging from cells, which are the primary pollinators of alfalfa seed (Pitts-Singer and Cane 2011). Historical trends of pathogens, predators, and parasitoids can provide insight into *M. rotundata* cell health and can provide valuable information on what can be considered as an acceptable baseline of infection for healthy bee stocks.

Materials and Methods

Parma Cocoon Diagnostic Laboratory Archived Samples

The PDCL is an extension-orientated service that classifies the proportion of pathogen- and parasitoid-infected *M. rotundata* cells submitted by growers (bees are submitted from growers stock, newly purchased bees from Canada or the United States, or growers submitting samples on behalf of another grower). Growers provide loose *M. rotundata* cells to the diagnostic laboratory. From each sample, five 10-g subsamples are weighed and X-rayed on a Faxitron (Model 42855A) machine using Kodak Industrex-M radiographic film, samples were processed at 20kVp for a 1-min exposure

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Fig. 1. X-ray of *M. rotundata* cells. Red highlighted cells indicate healthy bee cells, blue cells indicate chalkbrood, purple cells indicate predator or predator damage, and orange cells indicate parasites.

time (encompassing approximately 500 bee cells for each grower submitted sample) and the percent of cells affected by chalkbrood, parasites, and predators is calculated. The proportion of submitted nest cells affected by pathogens, parasites, and chalkbrood for each grower submitted sample (*n*~500 individual cells) represents a single datapoint within this analysis. Archived records ranged from alfalfa seed production areas located in North America including Idaho, Washington, Oregon, Montana, North Dakota, and Canada. The service visually classifies fungal pathogens including *Ascosphaera* infections, insect pests including imported chalcid wasps (*M. obscurus*), cuckoo bees (*E. pilosula*), woodboring chalcid wasps (*L. affinis*), long-tongued blister beetles (also known as sunflower beetles, *N. lutea*), Canadian chalcid wasps (*P. venustus*), and red-marked sapygids (*S. pumila*), and predators/nest destroyers including American black flour beetles (*T. audax*), giant flour beetles (*T. brevicornis*), and checkered flower beetles (*T. ornatus*) of cells using X-ray imaging (Fig. 1).

Diagnostic records of the incidence of chalkbrood, parasites, predators, and live larvae were compiled in 5-yr intervals from 1997 to 2021 (991 total samples, corresponding to 158 samples between 1997 and 2001, 28 samples from 2002 to 2006, 171 samples from 2007 to 2011, 317 samples from 2012 to 2016, and 317 samples from 2017 to 2021). A statistical analysis was performed in R version 4.1.2 (R Core Team, 2021) using functions available in the base distribution unless otherwise noted. Main effects tests were performed using analysis of variance (ANOVA). *Megachile rotundata* brood cell incidence proportions of chalkbrood, parasites, and predators were arcsine square root transformed prior to statistical analysis to satisfy assumptions of normality. The reported statistical significance of Year (5-yr period) in determining mean proportion of cells affected by chalkbrood, parasites, and predators was determined using analysis of variance of a fitted linear model with the formula *Response ~ Year + Grower* (farm operation that submitted the sample). Grower was included in the model to account for farm-specific effects and to account for different farms submitting samples across years. A post-hoc Tukey's HSD multiple comparison tests ($\alpha = .05$) were conducted using the R package 'agricolae' (Mendiburu 2021) to examine statistical differences between each of the 5-year intervals and to generate means separation letter codes.

Grower vs. Canadian Bee Stocks

In 2020, growers were asked to define whether the samples submitted were grower stock or newly purchased stock from

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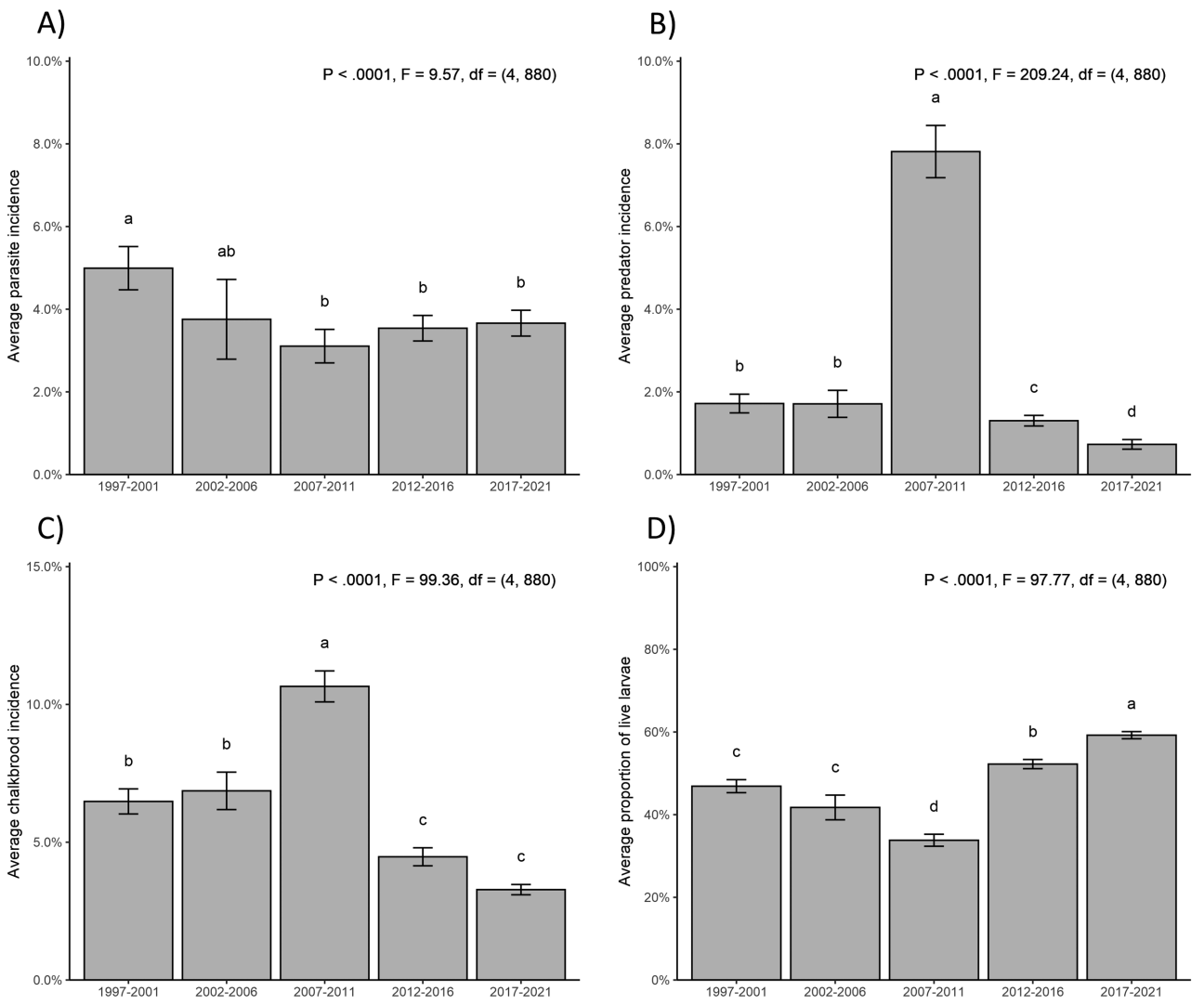

Fig. 2. Parma Cocoon Diagnostic Laboratory *M. rotundata* archived records. (A) Percent parasites (*M. obscurus, C. rufocaudata, L. affinis, N. lutea, P. venustus,* and *S. pumila*), (B) percent predators (*T. audax, T. brevicornis,* and *T. ornatus*), (C) percent chalkbrood (*A. aggregata and A. larvis*), and (D) live larvae. Significance of year was determined using ANOVA. Means separation letter codes were generated using Tukey's HSD test ($\alpha = 0.05$). Treatments that are not significantly different ($P > 0.05$) are designated by the same letter. Bars represent mean incidence of infected cells per 5-yr interval ± standard error.

Canada. Forty-nine samples received from growers were designated as grower stock or recently purchased bees from Canada (first-year stock purchased from Canada that have not been propagated in the United States and have not been placed in an incubator to complete development). This designation of stock source was used to evaluate the relative difference in chalkbrood, parasite, and predator incidence. Pairwise multiple-comparison t-tests were performed using the R package 'ggpubr' (Kassambara 2020) on a model incorporating *Stock source* and *Grower* as explanatory factors to identify statistically significant differences in affliction incidence by stock source.

Sex Emergence Ratio

Additionally, 105 archived records of the sex ratios of *M. rotundata* from 2010 to 2019 (excluding 2013) were analyzed depending on data availability (insufficient sex ratio datawere available for 2013). Pairwise t-tests were performed using the package 'ggpubr' (Kassambara 2020) on the model *Sex ratio* ~ *Year*, where *Sex ratio* is the square root transformed ratio of females to males.

Results

Parma Cocoon Diagnostic Laboratory Archived Records

Compiled records from the PDCL provide insight into the percent of chalkbrood, parasites, and predators found within *M. rotundata* cells processed at the diagnostic laboratory. The 5-yr average parasite incidence ranged from 3.10 to 4.99%. When the incidence of parasites found within brood cells was compared for each 5-yr interval, we noted that there was a significant difference between the timepoints we examined ($F = 9.57$; df = 4, 880; $P < 0.0001$; Fig. 2a). The 1997–2001 period had a significantly higher rate of parasite infection than any period from 2007 to 2021, with the intervening period 2002–2006 not significantly different from the earlier or later periods.

The incidence of predators found within brood cells also differed across multiple timepoints ($F = 209.24$; df = 4, 880; $P < 0.0001$; Fig. 2b) and ranged from 0.72 to 7.81%. The highest incidence rates of predator infecting brood cells were from 2007 to 2011. The 2007–2011 time interval also corresponded to the

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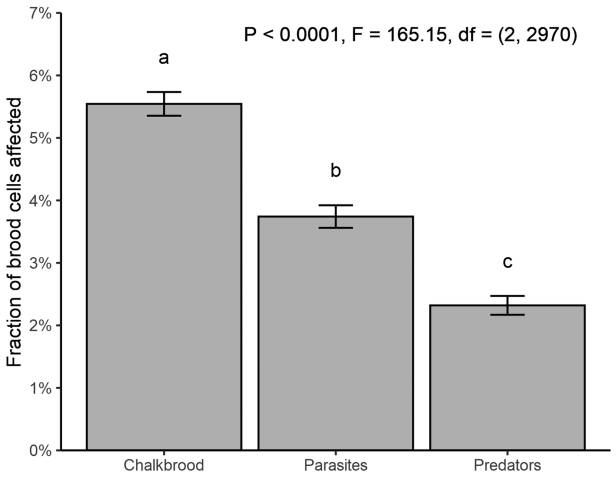

Fig. 3. Overall percent of chalkbrood, parasites, and predators within *M. rotundata* cells. Significance of year was determined using ANOVA. Means separation letter codes were generated using Tukey's HSD test ($\alpha = 0.05$). Treatments that are not significantly different ($P > 0.05$) are designated by the same letter. Bars represent mean incidence of infected cells ± standard error.

highest incidence rates of chalkbrood ($F = 99.36$; df = 4, 880; $P < 0.0001$; Fig. 2c). Overall chalkbrood incidence averages ranged from 3.27 to 10.65%. The 2007–2011 time interval also corresponded to the lowest proportion of live larvae ($F = 97.77$; df = 4, 880; $P < 0.0001$; Fig. 2d). We noted that percent of live larvae was also inversely correlated to the percent of chalkbrood and predators.

Within the 991 grower submitted samples examined, the highest percent of chalkbrood found within an individual sample was 39% in 2012, the highest percent for a single sample of parasites was 40% in 2012, and the highest percent of predators was 28% in 2009. When comparing the overall averages of chalkbrood, predators, and pathogens, all infection rates were significantly different from each other ($F = 165.15$; df = 2, 2972; $P < 0.0001$, Fig. 3), with average chalkbrood incidence highest (5.54%), followed by parasite infection (3.74%), and finally predation (2.32%).

Grower vs. Canadian Bee Stock

In 2020, we received 49 samples from growers to be examined for the presence of chalkbrood, parasites, and predators. From records provided by the growers, samples were designated as grower stock ($n = 43$) or newly purchased bees from Canada ($n = 6$). When we examined newly purchased bee cells from Canada and grower stocks, we observed that the Canadian bees had significantly less chalkbrood ($T = -2.78$; df = 11.53; $P = 0.017$) and predators ($T = -5.20$; df = 10.85; $P = 0.0003$), but that there was not a significant difference in parasites (Fig. 4).

Sex Emergence Ratio

Archived records of the sex ratio of emerged of *M. rotundata* adults were also analyzed from 2010 to 2019 based on available data (Fig. 5) to determine the ratio of female to male bees within the samples. Within all the years examined, there were statistically more male bees emerging than females, with the exception of 2011 and 2012 where the ratio was close to even. Overall, when all samples between 2010 and 2019 were combined, there were statistically ($T = 7.16$; df = 208; $P < 0.0001$) more males emerging in each sample (57.09%) compared to females (42.90%).

Discussion

The production of alfalfa seed is vital for the continued success of alfalfa hay and the livestock industry. Since alfalfa is partially self-incompatible, alfalfa seed producers rely predominately on two pollinator species, *M. rotundata* and *N. melanderi*. Unfortunately, *M. rotundata* brood cells can be preyed upon or play host to multiple different pathogens, parasitoids, and predators that can reduce the efficiency of grower bee stocks. Multiple investigations have explored and classified different fungal pathogens, insect parasites, and nest destroying beetles (Yousef et al. 1984, Goerzen 1991, Bisett et al. 1996, Wynns et al. 2012). While multiple different species that predate on *M. rotundata* cells have been determined, the presence of current, new, and different species that infest cells needs to be constantly surveilled, allowing growers and integrated pest managers to develop new approaches to keep bee stocks healthy. Further, James and Pitts-Singer 2013 demonstrated that geographic and management practices can significantly influence the presence of chalkbrood, parasites, and predators in the United States and that cleaning bee cells before storage can reduce the amount of pollen ball within grower stocks. While these management practices are important for pest control, we also need to examine historical records to determine expected, baseline percentages of pathogens, parasites, and predators. In the current investigation, we compiled archived records from the PDCL to reveal trends in the presence of common pathogens, parasites, and predators.

A principal goal of this investigation was to examine historical trends in the presence of pathogens, parasites, and predators from the PDCL to understand normal ranges and to better inform growers and pest managers of expected and appropriate concentrations of these different classifications within their *M. rotundata* stocks. The diagnostic laboratory examined 991 historical samples encompassing ~590,000 cells to examine trends in *M. rotundata* health. Examining yearly averages, we noted that there was between 3.27 and 10.65% of cells containing chalkbrood, 0.72 and 7.81% containing predators, and 3.10 and 3.75% containing parasites. These long-term averages can be used as a baseline for expected infection rates of samples and to inform growers regarding the health of their bee stocks.

Besides the average infection rates which provide insight for growers regarding expectations and cutoffs for future *M. rotundata* stocks, two important and significant observations can be drawn from this data. The first is that bees raised in Canada have a lower presence of chalkbrood and predators when compared to bees raised on U.S. farms that are propagated over multiple generations, even if the original stock was sourced from Canada. While these data were only collected over one growing season, the findings support the regular purchase of new bee stocks to maintain bee health. The second is that there were statistically more chalkbrood and predators in bee cells from 2007 to 2011. The Canadian dollar increased in value from 2002 to 2007 and stayed on par with the U.S. dollar though 2012 (Devereux 2009). The exchange rate would have significantly affected the price of *M. rotundata,* resulting in higher grower cost that may have indirectly resulted in U.S. growers purchasing fewer *M. rotundata* from Canada. We hypothesize that growers would have relied on more self-raised bee stocks over this time period and not subsidized their stocks with newly purchased bees to cut input cost, which may have resulted in higher infection rate of both chalkbrood and predators in bee cells.

Interestingly, the hypothesis that growers were producing more bee stock in the United States during 2007–2011 can also be linked to our sex emergence ratio findings. Pitts-Singer and James 2005 demonstrated that bees propagated in the United States generally

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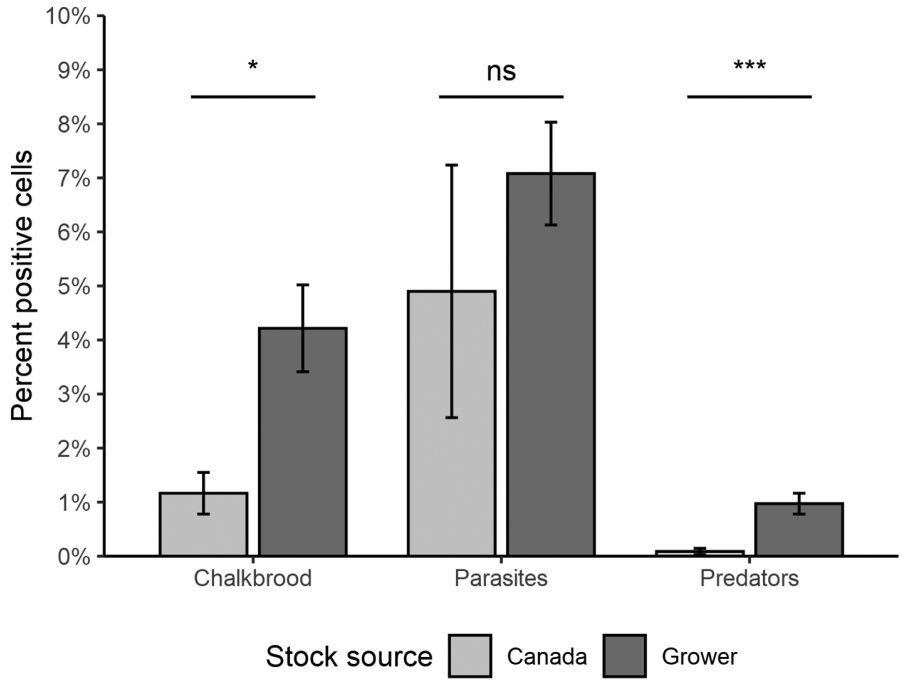

Fig. 4. Difference between chalkbrood, parasites, and predators within *M. rotundata* from Canadian first year samples and U.S. grower stocks in 2020. Significance was determined using pairwise T tests. Significant differences are denoted with a line between treatments and an asterisk (****P-values < 0.0001, ***<0.001, **<0.01, *<0.05). Bars represent percent positive cells from Canadian and grower stocks ± standard error.

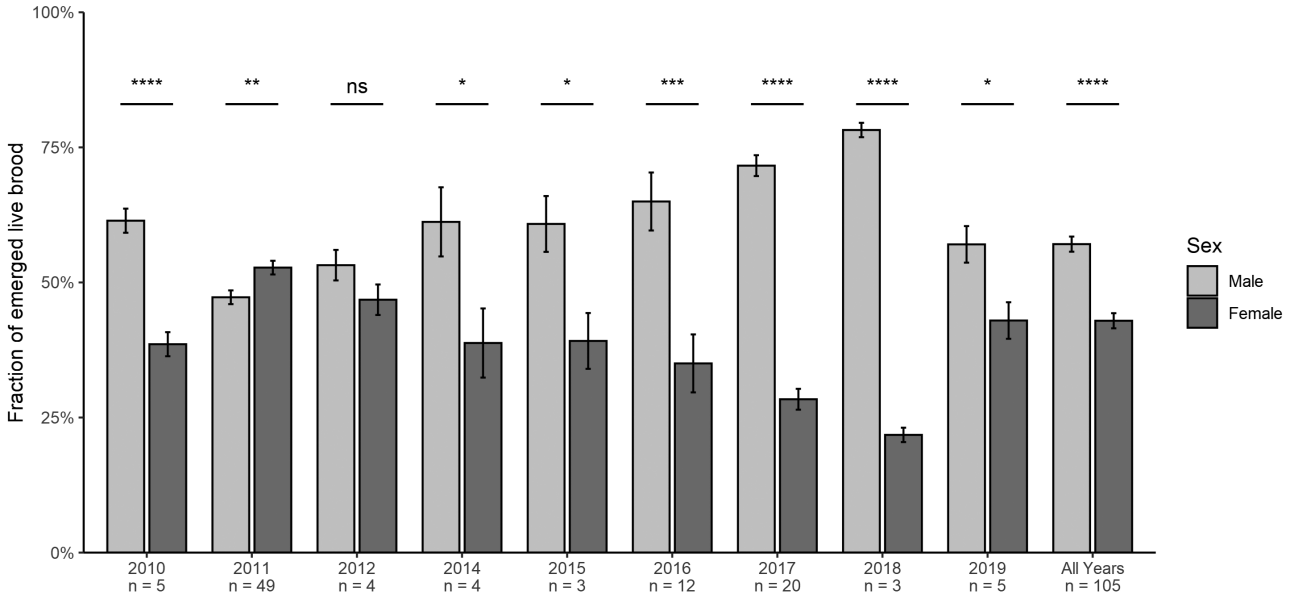

Fig. 5. *Megachile rotundata* adult sex ratio between 2010 and 2019. Yearly sex ratios that are significantly different are represented with asterisks. Significance was determined using pairwise T tests. Significant differences are denoted with a line between treatments and an asterisk (****P-values < 0.0001, ***<0.001, **<0.01, *<0.05). Bars represent mean fraction of male and female brood emerging yearly ± standard error.

have a 1 to 1 male to female sex ratio and can be female biased, while bees propagated in Canada are more likely to have a 2 to 1 male/female sex ratio (Pitts-Singer and James 2005). We noted that within all the years examined, there were statistically more male bees emerging than females, with the exception of 2011 and 2012. This observation reinforces our hypothesis that growers would be producing more bee stock in the United States, as grower produced stocks are predicted to have a higher male to female ratio. When we examined the presence of chalkbrood, predators, and parasites, we noted that in 2011 there was high proportion of predators (11.01%) and chalkbrood (10.93%) within this timeframe. When we compare this to 2018 (when the proportion of female bees was the lowest observed), we see a very low proportion of chalkbrood (2.35%) and predators (0.14%). While there are likely other environmental and management factors (nest tunnel diameter, nesting medium and tunnel length) that play a role in sex emergence, this correlation could suggest that the presence of chalkbrood and predators might play a role in the sex emergence ratio of bees.

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Within the current study, we determined expected presence of pathogens over all samples processed and found average infection rates of chalkbrood (5.54%), parasite (3.74%), and predators (2.32%), within historical samples analyzed at the PDCL. While these values are only averages, they can provide insight for growers regarding expectations and cutoffs to purchase future *M. rotundata* stocks. Knowing historical rates for percent of these pathogens and predators, growers can compare current bee stocks to historical samples. For example, the highest chalkbrood sample observed within this investigation was a 39% infection rate, well above the 5.54% average. Our extension recommendation would be to replace these highly diseased bees. Making these recommendations becomes more difficult when samples have infection rates closer to the average, but significant deviations are now easier to identify. In the current study, we did not investigate how infectivity rates affect pollination efficacy in the field. Future studies exploring pollination efficacy should be the focus of further investigation. As pollinators continue to be a vital resource for alfalfa seed producer, the agricultural community and growers should continue to monitor pollinator health and track trends in chalkbrood, predators, and parasitoids within *M. rotundata* stocks to make sure this important pollinating species remains a viable tool for alfalfa seed growers.

Acknowledgments

This research was supported by funding from United States Department of Agriculture Alfalfa Pollinator Research Initiative grant number 58-2080-0-009 awarded to J.C.

Ethical Statement

This article does not contain studies with any human participants and no specific permits were required for collection or experimental treatment of *Megachile rotundata* for the study described.

Data Availability

All relevant data are contained within the paper and its supporting information files.

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