Miticidal activity of fenazaquin and fenpyroximate against Varroa destructor, an ectoparasite of Apis mellifera

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Abstract

BACKGROUND: The Varroa mite (Varroa destructor) is an ectoparasite that can affect the health of honey bees (Apis mellifera) and contributes to the loss of colony productivity. The limited availability of Varroacides with different modes of action in Canada has resulted in the development of chemical resistance in mite populations. Therefore, an urgent need to evaluate new potential miticides that are safe for bees and exhibit high efficacy against Varroa exists. In this study, the acute contact toxicity of 26 active ingredients (19 chemical classes), already available on the market, was evaluated on V. destructor and A. mellifera under laboratory conditions using an apiarium bioassay. In this assay, groups of Varroa-infested worker bees were exposed to different dilutions of candidate compounds. In semi-field trials, Varroa-infested honey bees were randomly treated with four vetted candidate compounds from the apiarium assay in mini-colonies.

RESULTS: Among tested compounds, fenazaquin (quinazoline class) and fenpyroximate (pyrazole class) had higher mite mortality and lower bee mortality over a 24 h exposure period in apiariums. These two compounds, plus spirotetramat and spirodiclofen, were selected for semi-field evaluation based on the findings of the apiarium bioassay trials and previous laboratory studies. Consistent with the apiarium bioassay, semi-field results showed fenazaquin and fenpyroximate had high efficacy (>80%), reducing Varroa abundance by 80% and 68%, respectively.

CONCLUSION: These findings suggest that fenazaquin would be an effective Varroacide, along with fenpyroximate, which was previously registered for in-hive use as Hivastan. Both compounds have the potential to provide beekeepers with an alternative option for managing Varroa mites in honey bee colonies.

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Keywords: honey bee; Varroa mite; apiarium; fenazaquin; fenpyroximate

1 INTRODUCTION

Globally, many species of bees and other insects play an important role in plant pollination. This pollination service is vital to the maintenance of wild plant communities of the world1,2 and agricultural crop productivity.3,4 It is estimated that the value of insect pollination to global agriculture is $845 billion per year.5 Despite the wide diversity of plant pollinators, the honey bee, Apis mellifera L., is the world’s most important managed pollinator of agricultural crops and natural habitats.6,7 Approximately one-third of food consumed each day relies on pollination, mainly by honey bees.8 Klein et al.6 stated that the honey bee’s contribution to world food production is indispensable.

Similar to other managed agricultural species, particularly those kept in higher densities than would be seen in the wild, honey bees face a number of challenges for pest and disease management.10–12 Some maladies left untreated or without management intervention can cause colony mortality. Colony mortality has been associated with Varroa mite, Varroa destructor Anderson and Trueman,13,14 Nosema spp.,15 viruses,16–18 poor nutrition,19 and pesticides.20–21 Of the diseases and pests, Varroa have proven to be one of the most common causes of overwinter colony loss in Canada.22,23 Varroa mites feed on mature and immature honey bees, and failure to control high infestations can compromise individual bee health and overall colony viability. Parasitization from Varroa can suppress the normal functionality of many individual honey bee systems, including immune response,24 protein synthesis,25 lipid storage,26,27 and pesticide detoxification.28 At the colony level, Varroa can impede honey bee homeostasis29 and thermoregulation.30 Varroa mites are also responsible for...
Recently, Bahreini et al. screened the 16 active ingredients using the glass vial contact-based bioassay for miticide mortality and bee safety. To move to the next phase of developing new miticides to be used in honey bees, this study was designed to first screen the previously screened 16 compounds and additionally 10 more compounds using the apiarium bioassay, where a small cluster bees and mites together, similar to a colony environment. In addition to determining miticide efficacy in this novel laboratory environment, the goal was to see if this method would show comparable results in the field. If successful, the apiarium could be integrated into initial screening trials of new miticides, complementing conventional contact bioassays as a predictor of field efficacy. Second, vetted compounds from the apiarium test and the glass vial test were subjected to a field test using mini-colonies furnished with Varroa-infested bees.

2 MATERIALS AND METHODS

European honey bee (A. mellifera) colonies at the Crop Diversification Center North (CDCN), Edmonton, Alberta, Canada (53 54'N, 113.49'W) were used to provide Varroa mites and honey bees in the summer and autumn of 2017–2019. Management practices were implemented to reduce variables among V. destructor and A. mellifera populations. The experimental double brood-chambered colonies were headed by Kona queens (Hawaii, USA) and housed in Langstroth boxes. They were fed sugar syrup and treated, if needed, with Apivar (500 mg of amitraz/strip; Veto Pharma, Palaiseau, France), or oxalic acid and Fumagillin-B (both from Medivet Pharmaceutical Ltd, High River, AB, Canada) according to manufacturers’ recommendations, and were overwintered outdoors. To determine the Varroa mite infestation level (%) in each experimental colony, the initial mean abundance was evaluated using the alcohol wash method (70% ethanol). Evaluation of mite resistance to Apivar was performed on the colonies before mite collection using an adapted version of the Pettis method. This was done to ensure the mites used in the experiment were susceptible to amitraz, the AI for the Varroacide Apivar, which was used as the positive control. In the resistance test, 24-h mite mortality was assessed after a group of worker bees were exposed to a piece of Apivar strip. In all laboratory assessments, the temperature (°C) and relative humidity (RH, %) in incubators were monitored using HOBO (Onset Computer Corporation, MA, USA) data loggers.

2.1 Laboratory assessments

2.1.1 Chemical preparation

All AlS were obtained from Sigma-Aldrich (ON, Canada), except cfylumetofen (Cedarlane, NC, USA) (Table S1). Acetone (784 g mL−1 density; Sigma-Aldrich) was used as a solvent for all AlS, except for clofentezine where acetone (786 g mL−1 density; Sigma-Aldrich) was used. On the day of the experiment, fresh stock dilutions were prepared for each AI (10 000 mg L−1 = 1%) in 15-mL polypropylene centrifuge tubes (VWR, ON, Canada) using acetone or acetone, and agitated on a vortex mixer (VWR) for 3 min until homogeneous. A fume hood was used for the preparation of all chemicals and for the duration of the experiment. Operators wore full-face respirators (6900; 3M, USA) with filters (60 923; 3M) and additional personal protective equipment.

2.1.2 Apiarium bioassay

To determine the simultaneous effect of miticides on honey bees and Varroa, a group of adult worker bees (140.89 ± 6.37) from colonies with high mite infestations were transferred to a custom...
A plastic strip (2.54 cm; Recycled Binding Cover, Staples, Canada) coated with one of the experimental AIs was fixed to the top of the apiary with two sugar cubes on either side for bee food. In total, 19 chemical classes (26 associated AIs) with different MOAs, plus formamidine (amitraz), were tested and evaluated (Table S1). The efficacy of AIs for controlling *Varroa* mites and the potential lethal effect on worker bees was compared with a positive control (amitraz) and a negative control (no treatment). Three subsequent doses from each AI (*n* = 27, including amitraz) (0.05 mg/apiary, 0.5 mg/apiary or 5 mg/apiary) were evaluated. All treatment groups including controls had three replicates. After incubating for 24 h (25 ± 1°C and 60 ± 5% RH, dark), live and dead mites and bees were counted. Suspected dead mites were collected from the bottom of cages using a fine-tipped paint brush and probed under a magnifying glass to detect subtle limb movement. Mites that were completely motionless and lacked appendage movement when gently probed were considered dead. Bees were determined to be dead if they were laying on the bottom of the cage and completely motionless (i.e. lack of body or extremity movement) when the cage was slightly agitated. After dead bees were counted, cages were placed in a freezer at −20°C for 2–3 h to kill the remaining live bees. The total number of bees in the sample was counted and they were transferred to a 500-mL plastic container filled with 70% ethanol. The alcohol wash method was used to determine the remaining live *Varroa* left on live bees after 24 h.\(^{30}\) All experimental components in contact with chemicals (bees, mites, all parts of cages, contaminated alcohol) were disposed of appropriately.

### 2.2 Semi-field assessments

#### 2.2.1 Chemical preparation

Based on previous laboratory results by Bahreini et al.\(^{30}\) and the findings of the apiary in this study, four compounds were selected for semi-field evaluations. Formulated products (FPs) were used instead of the associated AIs (with the exception of fenazaquin), as more chemical was required to scale up to field testing and there is a higher procurement cost associated with the AIs. The tested FPs and AIs belong to the following chemical families: tetronic acids (spirodiclofen and spirotetramat), pyrazoles (fenpyroximate), and quinazolines (fenazaquin) (Table S1). All the commercial FPs were obtained from Terralink (BC, Canada): Kontos (spirotetramat, 22.4%), Fujimite (fenpyroximate, 22.4%), and Environ (spirodiclofen, 24%). The AI fenazaquin was used instead of its associated FP because it was not registered with Pest Management Regulatory Agency (PMRA) and is not available in Canada. On the day of in-hive application, fresh stock dilutions for each FP (10 000 mg L\(^{-1}\) = 1%) and AI (10 000 mg L\(^{-1}\) = 1%) were prepared in 50-mL polypropylene centrifuge tubes (VWR). The centrifuge tubes were agitated on a vortex mixer for a period of 2–3 min. All chemical preparations took place in a fume hood where operators wore full personal protective equipment.

#### 2.2.2 Mini-colony assay

Single brood chamber colonies (*n* = 14) were constructed with three separate compartments (12 × 26 × 48 cm). Each compartment was considered a single mini-colony with one brood frame, three frames of bees (for a total of three frames/compartment), and a newly mated queen. The origin of the frames and bees in each compartment corresponded to one highly *Varroa*-infested colony at CDCN (i.e. mother colony). No frames between mother colonies were mixed for this experiment. All mini-colonies (*n* = 42) were randomly assigned to treatments with three replicates. Miticide treatments were applied in three dosage levels: 500 mg AI/mini-colony, 1000 mg AI/mini-colony and 1500 mg AI/mini-colony. FP concentrations were calculated based on their labelled AI guarantee. Three mini-colonies were left untreated (negative control) and three mini-colonies were exposed to one strip of Apivar (500 mg amitraz/strip) as the positive control. Substrate strips (2.5 × 20 cm) were inoculated with dilutions of FPs or AIs representing designated dosages. Water and acetone were solvents for the FPs and the AIs, respectively. Prepared strips were air dried at room temperature (2–3 h) in a fume hood in the dark and applied to mini-colonies the same day. The experiment duration was 42 days, including a 28-day period for miticide exposure (treatments applied at 7-day intervals) and a 14-day post-treatment period. However, positive control mini-colonies had one treatment that lasted the duration of the experiment (i.e. one strip of Apivar for 42 days). To determine in-hive mite mortality, modified sticky traps (35.56 × 40.64 cm; Contech Inc., BC, Canada) were placed under the mini-colony and changed on the first, second, third, fifth, and seventh days after treatment (five total traps per week). Dead mites were double counted and the daily mite mortality rate (mites/total mites/day) and daily mite drop (mites/day) were calculated.

The mean abundance of *Varroa* was determined using the alcohol wash method for each mini-colony before and after treatments. The pre- and post-treatment bee populations were evaluated using visual assessment. The percentage of frames covered with bees was multiplied by 2430 worker bees.\(^{55}\) To determine the total number of *Varroa* mites remaining in the mini-colonies after treatment (28 days), excluding positive controls (Apivar), oxalic acid was used as a finishing treatment. Oxalic acid was applied using the Pro Vap 110 (Oxovap LLC, SC, USA) with modified application: 1 g per mini-colony, two treatments, 7-day interval between treatments. Sticky traps were also used to determine the mite drop during oxalic acid treatment. Dead mites were double counted and the remaining post-treatment mites in the bee cluster were calculated.

### 2.3 Statistical analyses

The variables for mite and bee mortality rates (%) were analyzed using a mixed model ANOVA (PROC MIXED) in which compounds were treated as main plots, dilutions or doses as subplots, mini-colonies and apiaries as experimental units, and replicates as random effects.\(^{36}\) Since the total numbers of *Varroa* mites were not equal in all replicates (laboratory or field assessments), a weighted statement was applied in analyses. In the laboratory assessment (apiary), mite and bee mortalities (%) were calculated based on the portion of dead mites or bees to total mites or bees in apiariums, respectively. Treatments (AIs) in the apiary assay were grouped based on average cumulative mite and bee mortality rates using a clustering method (PROC FASTCLUS). The effects of semi-field treatments on mite population were analyzed by ANOVA using a repeated measure analysis of variance using an autoregressive heterogeneous covariance structure with treatments as main effects, mini-colonies as subjects, and sampling periods as a repeated measure.\(^{36}\) Daily mite mortality rate (mites/total mites/day) was assessed based on dead mites collected from sticky traps placed under the mini-colony, length (day) of each sampling period, and initial total mites in the bee cluster using the equation:\(^{57}\):

\[
\text{daily mite mortality rate} = 1 - \left(1 - \frac{a}{100}\right)^{1/b}
\]
where \( a \) denotes the percentage of mite lost and \( b \) represents the length (day) of the sampling period. Total mites in mini-colonies was evaluated by adding all mites that dropped during the experiment to those collected during the finishing treatment. Length of sampling was the number of days that the sticky traps were in mini-colonies. However, cumulative mite drop (mites/day) in the semi-field assay was estimated as the sum of dead mites that were collected on the sticky traps over the length of the experiment divided by the total length of treatment (days). The arithmetic mean abundance of *Varroa* mites (%) in colonies was estimated using the alcohol wash technique to remove the mites from the bees and calculate the number of *Varroa* mites per 100 bees. For the change in mean abundance of mites, a before-after control impact design was used with mini-colonies as replicates, and the interaction between the main effects and the period was used as criteria to determine significant treatment effects. Percentage change in the adult bee population was calculated based on differences between the bee score before and after treatments using before-after control impact analysis. The Shapiro–Wilk test (PROC UNIVARIATE) was applied to test the normality of the data. The proportion for mortality rates that did not fit a normal distribution was arcsine transformed prior to analyses. All data are presented as untransformed values. Where significant interactions were observed, they were partitioned using the SLICE option in an LSMEANS statement and differences among treatment means were compared using Bonferroni correction. The efficacy of tested compounds in the semi-field trials was assessed based on mean abundance of mites in pre-treatment and post-treatment compared to negative controls using the equation:

\[
\text{efficacy} = 100 \left( 1 - \frac{Ta \times Cb}{Tb \times Ca} \right)
\]

where \( T_a \) and \( T_b \) indicate the mean abundance of mites at before and after treatment, respectively, in tested compounds, and \( C_b \) and \( C_a \) indicate the mean abundance of mites for negative control at the same time of treatment.

## 3 RESULTS

### 3.1 Laboratory assessments

Mite and bee mortality was assessed in the apiary, where *Varroa*-infested worker bees were exposed to plastic strips covered with subsequent dilutions of AIs (\( n = 26 \)) plus the positive control, amitraz. Cumulative bee mortality (\( F = 21.63; \text{df} = 27, 218; P < 0.0001 \)) and mite (\( F = 13.78; \text{df} = 27, 218; P < 0.0001 \)) mortality differed significantly among treatments. Results showed a higher 24-h bee mortality for higher dilutions of chlorfenapyr, emamectin benzoate, fenpropathrin, bifenthrin, and pyridaben compared to others (\( F = 34.7; \text{df} = 81, 164; P < 0.0001 \)).

Partitioning the interaction of the AI treatment \( x \) dilutions within each product indicated significant differences in 24-h mite mortality between different dilutions of amitraz (slice option: \( F = 3.87; \text{df} = 2, 164; P = 0.0228 \)), fenpyroximate (slice option: \( F = 8.66; \text{df} = 2, 164; P < 0.0001 \)), fenazaquin (slice option: \( F = 26.33; \text{df} = 2, 164; P < 0.0001 \)), tolfenpyrad (slice option: \( F = 5.63; \text{df} = 2, 164; P = 0.0043 \)), and pyridaben (slice option: \( F = 62.21; \text{df} = 2, 164; P < 0.0001 \)) or fenpropathrin (slice option: \( F = 28.86; \text{df} = 2, 164; P < 0.0001 \)). This showed that different dilutions of these AIs presented different mite mortality rates. Meanwhile, the cumulative rate of bee mortality was not significantly different among dilutions for amitraz, fenpyroximate or fenazaquin (\( P > 0.05 \)).

The cluster analysis divided the results of treatments into three clusters and the largest maximum distance between variables was...
found for the cluster 3 (Table 1 and Fig. 3). Therefore, based on the cumulative average mite and bee mortality (%) for each treatment (AI), the results of the apiarium bioassay were grouped into three categories: category 1 (n = 21) includes chemicals that had low mite efficacy (≤29%) and low bee mortality (≤20%), category 2 (n = 4) chemicals had high mite mortality (≥30%) and unacceptable honey bee mortality (≥21%), and category 3 (n = 3) chemicals had high mite mortality (≥30%) and low bee mortality (≤20%) (Figs 1–3 and Table S1). Using the results of the apiarium bioassay and our previous research for screening miticides with different MOAs, we selected four compounds (fenazaquin, fenpyroximate, spiридiclofen, and spirotetramat) to test on honey bee colonies under semi-field conditions.

### 3.2 Semi-field assessments

The results indicated a significant difference in cumulative daily mite mortality rate among treatments (weighted statement total mite: \(F = 139.07; \text{df} = 5, 36; P < 0.0001\)) (Table 2). Overall, repeated measure analyses indicated that the highest and most consistent daily mite mortality rate occurred for dilutions of Fujimite (500 and 1500 mg/mini colony), fenazaquin (1000 mg/mini colony), and Kontos (1000 mg/mini colony) compared to Apivar (weighted statement total mite: \(F = 58.43; \text{df} = 13, 28; P < 0.0001\)) (Fig. 4). Analysis of variance on the treatment × dose interaction was significant for formulated products (FPs) Envidor and Fujimite (weighted statement total mite: \(F = 58.43; \text{df} = 13, 28; P < 0.0001\)), meaning the effect of each treatment on daily mite mortality rate depended on the dose of Envidor (slice option: \(F = 3.50; \text{df} = 2, 28; P = 0.0441\)) or Fujimite (slice option: \(F = 5.38; \text{df} = 2, 28; P = 0.0105\)). Daily mite mortality rate during the trial increased after each treatment (Fig. 5). The greatest rate of daily mite mortality was observed for 500 mg Fujimite/mini-colony.

The cumulative average mite drop per day significantly differed among treatments (weighted statement total mite: \(F = 65.16; \text{df} = 5, 36; P < 0.0001\)) (Table 2). The mite drop was highest when colonies were exposed to all dilutions of Fujimite or fenazaquin (weighted statement total mite: \(F = 28.96; \text{df} = 13, 28; P < 0.0001\)) (Fig. 6). Although there was a significant interaction in dose × treatment (weighted statement total mite: \(F = 28.96; \text{df} = 13, 28; P < 0.0001\)), the cumulative daily mite drop was not dose-dependent for all tested compounds.

The cumulative mean abundance of mites was significantly different between pre- and post-treatments (\(F = 9.79; \text{df} = 5, 29; P < 0.0001\)) (Table 2) and for dilutions (\(F = 4.34; \text{df} = 12, 22; P = 0.0014\)). Among treatments, 1500 mg/mini colony of Envidor and all dilutions of Fujimite and fenazaquin along with Apivar reduced the mite abundance below the recommended autumn economic threshold (3%) (Fig. 7). Overall, cumulative mean mite abundance in some treatments significantly decreased by 96% (Apivar), 80% (fenazaquin) or 68% (Fujimite), compared to an increasing rate in the negative control (67%). For Kontos and Envidor, mean mite abundance slightly decreased by 30% and 20%, respectively. Among tested candidate compounds, the highest decrease (90%) in mean abundance was observed for fenazaquin at 1000 mg/mini-colony (Fig. 7).

The efficacy of the tested compounds was defined as a reduction in mean abundance of *Varroa* population (%) in treatments compared to the negative control over the experiment. The efficacy was significantly different among treatments (\(F = 5; \text{df} = 4, 32; P = 0.003\)) (Table 2) and within different dilutions (\(F = 3.52; \text{df} = 12, 24; P = 0.0042\) in mini-colonies). Analysis of variance presented a significantly high efficacy for Apivar, all dilutions of fenazaquin and Fujimite, and 1500 mg Envidor/mini-colony in comparison to others (Fig. 8). Partitioning the treatment × dose interaction indicated significant differences between the efficacy of doses only for Kontos (slice option: \(F = 2; \text{df} = 2, 24; P = 0.0158\)) and Envidor (slice option: \(F = 6.02; \text{df} = 2, 24; P = 0.0076\)). This indicates the other tested compounds presented similar efficacy within different doses (\(P > 0.05\)).

Bee populations significantly dropped over the duration of the experiment (\(F = 4.4; \text{df} = 5, 36; P = 0.0031\)). A significantly higher bee decline was observed in the negative control (74 ± 8%).

### Table 1. The results of clustering on apiarium observations

| Cluster | No. of treatments | No. of replicates | Frequency | RMS of SD | Maximum distance | Nearest cluster | Distance between cluster centroids |
|---------|-------------------|-------------------|-----------|-----------|------------------|----------------|-----------------------------------|
| 1       | 21                | 189               | 11        | 10.05     | 27.67            | 3              | 81.84                             |
| 2       | 4                 | 36                | 16        | 3.27      | 11.59            | 1              | 81.84                             |
| 3       | 3                 | 27                | 225       | 18.96     | 85.11            | 1              | 96.39                             |

The treatments \(n = 28\) based on the cumulative average mite and bee mortality in the apiarium assays were grouped into three clusters. RMS of SD is the root mean squared of standard deviation. The maximum distance is the maximum distance from cluster centroid to observation in each cluster.

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**Figure 3.** The scatter plot of *Varroa* mite and bee mortality in apiarium assay. Average cumulative *Varroa* mite mortality (%) was plotted against average cumulative bee mortality (%) in the apiarium assay. The symbols of red circle, blue triangle, and green square show distribution of treatments within each cluster. Each symbol represents one treatment \((n = 9)\). Dashed lines show the area for each cluster.

**Figure 2.** The scatter plot of *Varroa* population (%) in treatments compared to the negative control over the experiment. The efficacy was significantly different among treatments \((F = 5; \text{df} = 4, 32; P = 0.003)\) (Table 2) and within different dilutions \((F = 3.52; \text{df} = 12, 24; P = 0.0042)\) in mini-colonies. Analysis of variance presented a significantly high efficacy for Apivar, all dilutions of fenazaquin and Fujimite, and 1500 mg Envidor/mini-colony in comparison to others \((F = 4.4; \text{df} = 5, 36; P = 0.0031)\). A significantly higher bee decline was observed in the negative control (74 ± 8%).
However, the lowest decline rate was observed for Fujimite 500 mg/mini-colony (19 ± 7%) and fenazaquin 1000 mg/mini-colony (16 ± 8%), both significantly lower than the positive (48 ± 5%) and negative (74 ± 4%) controls (F = 3.07; df = 13, 28; P = 0.0062).

### 4 DISCUSSION

Considering the high cost of colony loss and risk of developing resistance to registered Varroacides already on the market, new products with a different MOA need to be assessed and developed for treatment of Varroa. This study took a different approach to screening new miticides by utilizing a newly designed laboratory bioassay method (apiarium) for testing mites and bees together, attempting to replicate a colony-like environment before field-testing. In this experiment, 26 AIs from 19 chemical classes were screened in the laboratory using plastic strips, similar to Varroacides such as Apistan, Apivar and Bayvarol. Overall, the results of the apiarium and semi-field trials confirmed fenazaquin as a potential Varroacide, followed by fenpyroximate. Both products had high efficacy (>80%), reduced the mite population below the recommended autumn economic threshold (3%), and met the initial criteria for further testing. The preliminary results from this study are foundational for the development and registration of a new Varroacide; however, we do not recommend the off-label application of any miticide or AI used in this study before extensive testing is completed and safety in hive products are determined based on the final application method.

A number of investigations have exposed Varroa to miticides in glass vials, but few have tested bees and mites together. The apiarium provided an opportunity to test bees and mites simultaneously, while evaluating the application method that would be used in field trials if chemicals had acceptable results. Three categories were created based on the apiarium results to help in vetting compounds for field-testing. Compounds in category 1 showed low mite mortality and low bee mortality, and were excluded from field trials, with the exception of spirotetramat and spirodiclofen, which demonstrated high mite mortality in the Bahreini et al. contact-bioassay trials. One compound in this category, spiromesifen, had been previously reported to have high efficacy (approximately 80%) when mites were exposed to the FP (Forbid, Bayer, Canada) in contact-bioassays using glass vials. These results were not replicated when tested in contact bioassays or in the apiarium bioassay with the AI. To see higher efficacy, this compound may require more time (>24 h, chronic effects), higher doses, or changes to the application method. Some dilutions of miticides tested in category 2 (e.g. bifenthrin, fenpropatrin, and pyridaben) showed high mite mortality after 24 h, similar to amitraz (positive control), but higher dilutions of the AIs killed an unacceptable amount of bees. Although harmful to bees at the tested dilutions, they should not be excluded from future testing. Opportunities exist to examine different doses and application methods in a longer duration to evaluate chronic effects. Our study showed bifenthrin caused high mortality in bee clusters similar to Ellis et al., but lower dilutions killed >90% mites in the apiarium test. Future studies may seek to find an effective dose of bifenthrin for mites that is tolerable for honey bees. Precautions should be taken as this compound is in the same class as tau-fluvalinate and flumethrin (pyrethroids), and there is a high risk of cross-resistance. Additional compounds

### Table 2. Mean (±SE) cumulative daily mite mortality, daily mite drop, mean abundance, and efficacy of tested compounds in mini-colonies

| Treatment (AI)          | Daily mite mortality rate (mites/total mites/day) | Daily mite drop (mites/day) | Changes in mean abundance (%) | Efficacy (%) |
|-------------------------|-------------------------------------------------|-----------------------------|-------------------------------|-------------|
| Apivar (amitraz)        | (9.3 ± 2.3)*10^2 ab                             | 10 ± 1.4*cd                 | -96a                          | 98a         |
| Negative control        | (0.08 ± 2.2)*10^-2 c                            | 4.3 ± 1.4*cd                | 67c                           |             |
| Envidor (spirodiclofen) | (5.9 ± 1.5)*10^-2 b                            | 6.2 ± 1.9*cd                | -20b                          | 20a         |
| Fenazaquin              | (10.3 ± 1.3)*10^-2 ab                           | 30 ± 8.2*bc                 | -80a                          | 88a         |
| Fujimite (fenpyroximate) | (12.4 ± 1.1)*10^-2 a                          | 54.6 ± 6.6*a               | -68ab                         | 80ab        |
| Kontos (spirotetramat)  | (9.4 ± 1)*10^-2 ab                              | 20.5 ± 6.6c                | -30b                          | 53b         |

### Figure 4. Daily Varroa mite mortality rate in experimental mini-colonies.

Mean (±SE) daily mite mortality rate (mites/total mites/day) in mini-colonies were treated with different doses (500 mg/mini-colony, 1000 mg/mini-colony or 1500 mg/mini-colony) of FPs (Envidor (spirodiclofen), Kontos (spirotetramat), Fujimite (fenpyroximate)) or Al (fenazaquin). Additional sets of three mini-colonies were treated with Apivar (positive control) or left untreated (negative control). Means with the same letter in each column among treatments were not significantly different (P > 0.05).
in category 2, fenpropahrin and pyridaben, lack information on the effects on V. destructor or A. mellifera. Despite having high mite mortality in the apiarium bioassay, fenpropahrin and pyridaben also killed an unacceptable amount of worker bees. More sensitive testing could identify a dilution that is safe for bees and effective for controlling mites. Category 3 compounds (fenazaquin and fenpyroximate) showed high mite mortality and low bee mortality in the apiarium, and were selected for testing under semi-field conditions.

Our experiment was the first investigation to evaluate spirotetramat and spiropdiclofen (category 1 compounds) on the effects on V. destructor and A. mellifera under semi-field conditions. These compounds were selected for field evaluation as they performed well in the Bahreini et al. contact bioassay experiments (glass vial test), but demonstrated low efficacy on mites in the apiarium bioassay. The purpose of including these two chemicals in field trials was to observe and evaluate whether the contact-bioassay or the apiarium would be a better predictor of field-level efficacy on Varroa. Spirotetramat and spiropdiclofen are tetroic acids with inhibitory effects on lipid biosynthesis. There are a number of tetroic acid miticides currently registered in Canada and widely used against a broad range of pest insects and phytophagous mites. Miticides in this chemical family provide various options for potential Varroa control, but many studies indicate the presence of tetroic acid resistance in Panonychus ulmi (Koch), Panonychus citri (McGregor), and T. urticae Koch. T. urticae, for instance, has the ability to resist new miticides within a few treatments due to innate detoxifying mechanisms. It has not been confirmed if these mechanisms are present in Varroa mite populations, therefore close monitoring is essential if tetroic acids are chosen for future evaluation. Spirotetramat has been recognized as a relatively safe AI for A. cerana indica Fab. compared to other pesticides, but A. mellifera mortality has been reported from colonies exposed to spirotetramat from crop and tree applications. In previous laboratory tests, both spirotetramat and spiropdiclofen had positive, dose-dependent bee mortality in the Mason jar bioassay. In contrast, the results of the apiarium and field studies showed both compounds had low bee mortality. This may be due to different exposure methods. Although our semi-field study showed commercial FPs of spirotetramat (all dilutions of Kontos) and spiropdiclofen (lower dilution of Envidor) had low efficacy for reducing mean mite abundance, testing different doses of the AI or different application methods could yield higher efficacy for reducing Varroa populations.

Of the two most promising miticides from the apiarium experiment, Fujimite (fenpyroximate) showed the second highest reduction in mean abundance of Varroa in semi-field trials (80% efficacy). Fenpyroximate is from the pyrazoles chemical class and was commercialized in 1991 by Nihon Noyaku. It inhibits mitochondrial electron transport at the nicotinamide adenine dinucleotide (NADH)-coenzyme Q reductase site of Complex I with low to moderate effects on beneficial insects and predatory mites. Fenpyroximate was previously introduced in 2007 as a commercial Varroacide (Hivastan, Wellmark, USA) in the USA to control V. destructor, but was not registered in Canada. Hivastan was formulated in a patty containing 0.3% fenpyroximate.

Figure 5. Variability in the rates of daily mite mortality in treated bees. Mean daily mite mortality rate (mites/total mites/day) in mini-colonies treated with different doses: 500 mg/mini-colony (solid triangle), 1000 mg/mini-colony (solid square) or 1500 mg/mini-colony (solid circle) of the FPs [Envidor (spirodiclofen), Kontos (spirotetramat), Fujimite (fenpyroximate)] or AI (fenazaquin). Green arrows represent the time points of treatment application. Each symbol (solid square, circle or triangle) indicates the average daily mite mortality rate for all replicates in each dose (n = 3). Each time point of sampling represents the nth day of sampling (i.e. first, second, third, and so on).

Figure 6. Daily Varroa mite drop in treated bees. Mean (±SE) daily mite drop (mites/day) in colonies that were exposed to different doses (500 mg/mini-colony, 1000 mg/mini-colony or 1500 mg/mini-colony) of FPs [Envidor (spirodiclofen), Kontos (spirotetramat), and Fujimite (fenpyroximate)] or the AI (fenazaquin). Additional sets of three colonies were treated with Apivar (500 mg amitraz per strip, positive control) or left untreated (negative control). Each symbol represents average mite drop for replicates of each dilution (n = 3) or control treatments (n = 3). Vertical lines on each symbol indicate ± standard error (SE). Means with the same letter among dilutions were not significantly different (P > 0.05).
Since the FP of fenazaquin (P450 and ester activities). Honey bees are exposed to spider mites (Tetranychus spp.), but resistance to fenpyroximate has been reported in some target species. 

Our laboratory results showed slightly higher, but not significant, difference in bee mortality comparing fenpyroximate and amitraz. In contrast, Johnson et al.83 found higher toxicity for amitraz than fenpyroximate. The difference in toxicity could have resulted from the application method or the dose applied, as Johnson et al.83 directly exposed bees to the AI and held them in wax-coated paper cups (177 mL). Another study reported that fenpyroximate had a higher toxicity to worker bees than queens; however, queen health was still impacted at very low doses over a 6-week period.81 In our semi-field experiment, we did not see a negative acute effect of fenpyroximate on queens, which is likely attributed to the short experiment duration. Unlike previous laboratory studies,83 our semi-field results also show that the small bee cluster in the mini-colonies can tolerate up to 1500 mg over a 4-week period, while showing high efficacy for Varroa control. Although fenpyroximate is not currently used in bee colonies, it has been used for other species. Fujimite is currently used as a tool against spider mites (Tetranychus spp.), but resistance to fenpyroximate has been reported in some target species.84–87 We propose that fenpyroximate should be reassessed and potentially reformulated for use against Varroa for in-hive use. Further studies to find an effective formulation, dose, and application method are required. Once found, a longer study would be necessary to test the chronic influences of this Varroacide on full-size colonies to investigate any sublethal effects on queen physiology and colony fitness.

Fenazaquin had the highest efficacy to control Varroa in the semi-field study. It is in the quinazolines chemical class and was introduced by Dow AgroSciences in 1998.80 Since the FP of fenazaquin (Magister, Gowan, AZ, USA) was not available in Canada, we used the AI in semi-field trials. The quinazoline class includes chemical compounds representing a wide variety of biological activities against animal and human parasites and pathogens.91 Quinazoline commercialized FPs have reported minimal impacts on many beneficial insects and mites.92 Fenazaquin inhibits the mitochondrial electron transport chain and has been used against different stages of spider mites.93 At present, there have been no reports of Varroa resistance to fenazaquin; however, it is important to note that P. ulmi84 and T. urticae95 were documented as fenazaquin resistant. To our knowledge, this study is the first investigation testing the efficacy of fenazaquin on V. destructor and the effects on A. mellifera. Additionally, no documented studies have evaluated the potential risks of fenazaquin on honey bee physiology, behavior or fitness. The results of this study showed that fenazaquin decreased the mean abundance of Varroa by 88% with no observed negative effects on the bee population. Further research is required to evaluate fenazaquin along with its associated FPs in full-size Varroa-infested honey bee colonies, as commercial miticides contain adjuvants and synergistic compounds that can inhibit compounds (e.g. pyrazoles chemical class) in agricultural environments. The presence of pesticides in bee-collected foods or agrochemical residues in wax may contribute to developing Varroa resistance to pesticides. Despite the potential for resistance development in Varroa, proper integrated pest management (IPM) practices, such as rotation of available treatments, can delay resistant genes from becoming prevalent.88,89
enhance their efficiency.\textsuperscript{96} We strongly suggest that fenazaquin be considered as a potential new Varroacide and recommend more stringent studies to complete our investigations before commercialization. There is potential for Varroa to develop resistance to fenazaquin, similar to fenpyroximate, nevertheless, the resistance mechanism is not known and should be determined. As always, proper IPM integration of any chemical pesticides should be followed to suppress resistant pest populations. Also similar to our suggestions for fenpyroximate, a fully formulated application method and longer experiment duration (chronic and sublethal effects) would be required before attempting to register any products for in-hive use against Varroa.

One of the objectives of this study was to determine whether the apiary could capture the miticide-Varroa-honey bee interaction, similar to what would be observed under semi-field conditions, when compared to the contact vial bioassay. Despite being a preliminary study, our results show that there is a relationship between miticide efficacy in the apiary in the laboratory and semi-field trials. Fenazaquin and fenpyroximate, for instance, performed well in the contact bioassays\textsuperscript{97,98} and in the apiary, and showed the highest efficacy at the colony level in semi-field trials. Meanwhile, spiromesifen and spiroetramat performed well in contact bioassays, poorly in the apiary, and showed minimal effectiveness in the semi-field trials. More investigations are needed, but results are encouraging that the apiary can be a useful tool for screening chemicals before scaling up to field assessments. As with any bioassay method, there are factors that limit the apiary's efficacy. Previous research suggests that Varroa parasitization shortens bee longevity and reduces the honey bees' ability to process pesticides due to their compromised immune system.\textsuperscript{28} This could explain some of the variation in bee mortality between dilution replicates because each apiary's mite infestation level was not equalized. Apiaries with higher mite abundance may experience increased bee mortality rates, not necessarily due to the compound introduced, but due to a compromised immune system caused by Varroa parasitization. The semi-field trials also had some limiting factors that should be addressed. The delivery method may have restricted the amount of chemical released over the experiment. Other factors that must be considered in Varroacide screening field trials are the local environment and genetics of tested Varroa mites and honey bees. Field tests of chemicals differ from laboratory trials due to a larger environment, uncontrolled ambient environmental elements (e.g. temperature, humidity, and CO\textsubscript{2}), and social interactions among individual bees. This can influence the interaction between miticide-Varroa-honey bees and needs to be considered for field trials. Furthermore, Varroa populations with previous exposure to compounds in a similar chemical class could have pre-existing resistant genetics (cross-resistance) and influence results. Additionally, resistant bee stocks with grooming genotypes could reduce the Varroa phoretic population through defensive behavior,\textsuperscript{97–99} which can be mistaken for miticide efficacy.

5 CONCLUSION

Current treatments available to beekeepers for Varroa control are limited and there is growing evidence that resistance is developing in the few registered miticides for use by beekeepers.\textsuperscript{22,35,36} Inability to control Varroa populations not only threatens colony health, it can lead to substantial economic losses for beekeepers and crop pollinations. Therefore, finding new Varroacides is paramount for the health of the beekeeping industry. The purpose of this research was to expand on our previous study\textsuperscript{50} and conduct further evaluation of identified compounds under laboratory and field conditions. Based on our current results we have identified fenazaquin (quinazoline class) and fenpyroximate (pyrazoles class) as potential compounds that could be developed for in-hive use against V. destructor. According to the pesticide registration system in Canada, pesticides are considered as ‘effective’, if their efficacy is above 80%.\textsuperscript{65} These compounds with >80% efficacy and safe to honey bees are good candidates for future registration in Canada. Although our research presents advancement towards commercial product development, further rigorous testing of dosage, method of application, and safety to hive products and applicators needs to be completed to meet all requirements for legally registered use in honey bee colonies. Moreover, it has yet to be determined how these chemicals influence all A. mellifera or V. destructor developmental stages, and if there are sublethal or chronic long-term colony affects. Furthermore, these compounds need to comply with industry regulations for low residues in bee-related products (e.g. honey, wax, propolis, and pollen) and be safe for the applicator. Overall, fenazaquin and fenpyroximate both have the potential to add significant economic value to apiiculture and agriculture if they align with all industry and federal regulations for pesticides. Additionally, both compounds could provide effective Varroa control and alternative options for managing Varroa resistance to be included in current IPM practices, and enable sustainable, productive, and healthy honey bee stocks for the future.

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CONFLICT OF INTERESTS

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

Conceptualization: RB, MN; formal analysis: RB; funding acquisition: MD, DF; investigation: RB, CD, OD, SM, MN; methodology: RB, MN; writing – original draft preparation: RB, CD, OD; writing – review & editing: RB, CD, OD, SM, MN, DF.

DATA AVAILABILITY STATEMENT

Data are available on request.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.
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