Project 2014C004R: Increased Sustainability Managing Bee Pests (*Varroa* Mites and *Nosema*) for Alberta Beekeepers

Section A: Project overview

1. **Project number**: 2014COO4R
2. **Project title:** Increased Sustainability Managing Bee Pests (*Varroa* Mites and *Nosema*) for Alberta Beekeepers
3. **Research team leader**: Dr. Shelley Hoover / Dr. Medhat Nasr
4. **Research team leader's organisation**: Alberta Agriculture and Forestry
5. **Project start date**: (2014/04/1):
6. **Project completion date:** ACIDF (2017/12/31)
   Amendment: Alberta Beekeepers (2018/12/31)
7. **Project final report date:** ACIDF (2018/01/31)
   Amendment: Alberta Beekeepers (2019-02-01)

Section B: Non-technical summary

Honey bees are an integral part of our agroecosystems, providing essential ecosystem services from pollination that contribute between $4 - $5.5 billion to the Canadian economy each year (Mukezangango and Page 2017). Alberta is the leading apicultural province in Canada, with more than 315,000 colonies (over 40%) of the total of 750,000 honey bee colonies across Canada (Mukezangango and Page 2017). From 2006 to 2011, Alberta beekeepers reported over-wintering colony losses as high as 44%, nearly 3x the historical long term average of 15% (Hartman and Nasr 2008). These losses alarmed industry stakeholders and highlighted the risks to beekeeping businesses and honey bee health. In addition, public interest in the health of honey bees in particular, and bees in general has been heightened by the increase in over winter losses. To determine the causes of high winter kill, 112 beekeepers with greater than 400 colonies were surveyed in 2008. From this survey, in addition to winter weather conditions, high winter mortality in Alberta was attributed primarily to the presence of two parasitic species on honey bees in Alberta: the *Varroa* mite (*Varroa destructor,* an ecto-parasite) and *Nosema* (*Nosema apis* and *Nosema ceranae*, intestinal parasites). Options for controlling both of these parasites are limited to one or few treatment options, placing the industry at risk.

The only highly effective chemical *Varroa* mite control currently available is amitraz. Other registered chemicals are no longer effective in Canada due to the development of resistance to these compounds in the mite population (Currie et al. 2010), or due to being chemically related to compounds with documented resistance (e.g. Bayvarol®). Unfortunately, resistance to amitraz has also been documented in other countries around the world (Thompson et al. 2002), and there has been suggestions of resistance to this product in Alberta and other Canadian Provinces. It is not a matter of *if* resistance to amitraz will develop in Alberta *Varroa* populations, but rather *when* it will develop, and what other tools will be available to Alberta beekeepers to control this parasite when this occurs.

The only registered chemical treatment for *Nosema* was the antimicrobial fumagillin (Fumagilin-B®, Medivet Pharmaceuticals), but this was taken out of production in 2018, leaving beekeepers with no chemical treatment options. Without effective control of *Varroa* and *Nosema*, winter colony losses will continue to be high, threatening the viability of beekeeping operations in Alberta, and the ability of beekeepers to provide an adequate number of healthy colonies for honey production and the pollination of hybrid canola seed fields, fruit, and forage crops. The aim of this project was to identify and evaluate alternative pest and disease control options.

For *Varroa* mite control, 22 new candidate miticides were first screened in the laboratory. The 9 miticides that showed high efficacy for controlling *Varroa* mites and low honey bee mortality in the lab were tested in field trials. Two candidate compounds (Pyrazole I and Quinazoline) showed promising results, but further testing is required before the products can be submitted for registration with Health Canada. In addition, field tests of two existing *Varroa* mite treatments, oxalic acid and HopGuard were carried out. Oxalic acid which is currently available to beekeepers, was tested with two new application methods, using either blue shop towels or cardboard strips. Our results were promising, but further

testing is required before the new application methods can be submitted for registration with Health Canada. HopGuard, which is currently only available in the USA, underwent extensive modifications and field testing. It was subsequently submitted to Health Canada for registration as HopGuard II. Finally, a pilot study was completed to evaluate the efficacy of commercial oxalic acid vaporizers on mite mortality. Final results from this study will be available mid-2019.

Eleven new chemicals for the treatment of *Nosema* spp. were screened in the laboratory and one in a field trial. This series of experiments has identified Artemisinin as a promising alternative to fumagillin. In addition, seasonal variation, wintering method, and the effect of treatment timing on the abundance of *Nosema* spp. in honey bees was studied in southern and northern Alberta. This study will be complete in 2019, and results will be used to improve efficacy of treatments and management recommendations.

Numerous extension and outreach activities were delivered to beekeepers and stakeholders to improve their management of honey bee colonies. As a part of outreach activities, a mobile app "Bee Health" was developed to aid beekeepers in pests and diseases diagnosis, treatment, and reporting (sponsored in part by *Growing Forward 2*, a federal-provincial-territorial initiative). These extension activities are critical to ensure healthy bees and the long-term sustainability of the beekeeping industry in Alberta.

Section C: Project details

1. Project team

Team Leaders Dr. Medhat Nasr, Provincial Apiculturist was instrumental in proposing and developing this project. He assembled the research team to execute this research, and worked closely with the research team to ensure all studies were conducted according to plan and to solve problems arising throughout the research. He also carried out the outreach program with the bee team to improve beekeepers skills and expand awareness of public regarding bee health across Alberta and Canada. Upon Dr. Nasr's retirement, **Dr. Shelley Hoover** assumed responsibility for this project as of summer 2018. Dr. Hoover ensured the project continued as outlined, and compiled this final report with assistance from the project team.

Team Member Dr. Rassol Bahreini, Research Scientist, was hired to contribute substantially to this project. With the assistance of staff at the Crop Diversification Centre North, Dr. Bahreini was essential to developing assays and carrying out all screening tests of potential miticides to control *Varroa* mites and antibiotics for treatment of *Nosema*, as well as studies with oxalic acid. With other research staff, he performed all necessary lab and statistical analyses, and drafted reports on screening test results.

Team Member Dr. Robert Currie: Professor, University of Manitoba, supervised the M.Sc. thesis of Rosanna Punko on *Nosema* (objective 3) in the Entomology Department at the University of Manitoba. He contributed to *Nosema* research project plans and will continue to supervise Rosanna's research and degree.

Team Member Ms. Samantha Muirhead: Senior Technologist / Acting Provincial Apiculturist at Alberta Agriculture and Forestry, was essential to supervising the bee team and preparing bee colonies for research, and analyses of samples. She trained staff and executed experiments in the field, gathered data, and contributed to the drafting of reports.

Team Member Dr. Tom Thompson: Research Scientist, Alberta Agriculture and Forestry determined HopGuard residues in honey samples.

Bee Research Technical support: The following are names of technicians and beekeepers who contributed substantially to the project. Without their assistance, this work would not have been possible: Gerard Sieben, Reece and Echo Chandler, Cassandra Docherty, Olivia Hares, Michelle Fraser, Mellissa Howard, Maksat Igdyrov, Rosanna Punko, Alexandra Panasiuk, Karlee Shaw, Glyn Stephens, Sian Ramsden, Sarah Waterhouse, Emily Olson, Eric Jalbert, Jeff Kearns, Parisa Fatehmanesh, Jared Amos, Paul Schmermund, and Derek Rennie

2.Background

Alberta beekeepers continue to be vulnerable to high bee losses due to a variety of contributing factors. In the winter of 2017/18, winter losses across the province averaged 34%. These high winterkills are double the normal long term loss average of 15%. Despite research suggesting that there are many contributing factors (pests, parasites, nutrition, stress, pesticides) causing losses in the USA, Canadian losses have been primarily attributed to failure to control *Varroa* mites and *Nosema* (microsporidian pathogen), queen quality, and inclement winter conditions (Canadian Association of Professional Apiculturists 2018).

In Alberta, the estimated cost of 30% honey bee colony winter losses in 2007 was up to $25 million (Chaudhary and Nasr 2007). More recent estimates of the cost of loss of production and replacement of 30% winter losses could be up to $70 - 75 million/year (Laate and Nasr 2013). These high annual colony losses impact the sustainability of the beekeeping industry itself, as well as the ability of beekeepers to meet demands for pollination in both BC and Alberta.

Currently, the primary chemical control for *Varroa* mite management in Alberta is the product Apivar®, active ingredient amitraz, and no highly effective strip alternatives are available. Unfortunately, *Varroa* resistant to amitraz have been reported from countries around the world (Elzen et al. 2000, Kamler et al. 2016). In Alberta, beekeepers have been using Apivar® for over 8 consecutive years as a critical part of their management program. This repeated use of the same miticide contributes to the development of resistance of *Varroa* mites to Apivar®. Indeed, some Alberta beekeepers have already reported lower than expected efficacy of Apivar®, although resistance has not been confirmed. Investigation of these cases showed that Apivar® resistance may be emerging in Alberta. If Apivar® resistance continues to develop and spread across Alberta, the industry will be unable to effectively control mites and high bee mortality will be expected. This is a serious risk that demonstrates the vulnerability of the beekeeping industry. *Varroa* mite management is the most serious threat to bee health and survivorship in Alberta.

The Alberta Agriculture and Forestry Apiculture Unit continues to monitor the efficacy of Apivar® (amitraz) across Alberta. We have also revised and focused the objectives of this project to prioritise the development of alternative control measures for *Varroa* mites using new chemicals with unique modes of action.

Nosema is a genus of microsporidian parasite, with two species that infect honey bees in Alberta. It is considered a serious disease in the Northern Hemisphere, including all of Alberta. The Apiculture Unit research program has also focused on researching *Nosema* treatments and management options to develop alternatives to fumagillin (Fumagilin – B®). Beekeepers have had access to only a single antibiotic, fumagillin for treatment of *Nosema* for the past 50 years. If *Nosema* develops resistance, beekeepers will be left without an effective medication to treat *Nosema*. Studying seasonal variation in *Nosema* infection rates and prevalence in Alberta and determining appropriate timing for applications of fumagillin is important to improve our management recommendations to

beekeepers. Furthermore, screening antibiotics for treatment of *Nosema* is another important aspect of this research.

The main objectives of this project were to improve the sustainability of the beekeeping industry by providing beekeepers with alternative options for controlling resistant strains of *Varroa* mites and *Nosema*. The research included in this project included screening new chemical compounds for activity against *Varroa* and *Nosema* and for bee safety, examining the contributions of treatment timing, geography, and overwintering management to the epidemiology of *Nosema*, and refining application methods for two registered miticides: HopGuard and oxalic acid.

3. Objectives and deliverables

Original objectives

1. Continue developing alternative new miticides to enhance the rotation system for sustainable control of resistant *Varroa* mites:
   (a) Semi-field and full scale field testing the efficacy of three identified miticides
   (b) Evaluation of safety on bees and honey
2. Assess the treatment time effects on *Varroa* populations, levels of different viruses found in bee colonies in relation to annual winter mortality
3. Evaluation of factors affecting *Nosema* outbreaks and annual winter mortality
4. Screening alternative antibiotics for viable *Nosema* treatment

2017 Modified Project Objectives

1. Developing alternative new miticides to enhance the rotation system for sustainable control of resistant *Varroa*
   (a) Lab screening of 22 compounds
   (b) Evaluation of safety on bees
   (c) Semi-field and full scale field testing of promising compounds that demonstrate efficacy against *Varroa* and bee safety in lab trials
2. Developing alternative new treatments to enhance the rotation system for sustainable control of resistant *Nosema*
   (a) Lab screening of 11 compounds
   (b) Evaluation of safety on bees
   (c) Semi-field and full scale field testing of promising compounds that demonstrate efficacy against *Nosema* and bee safety in lab trial
3. Evaluation of factors affecting *Nosema* outbreaks and annual winter mortality (*this original objective was re-added into the present report despite having been removed from the 2017 modified objectives as we do have some in-progress results to report on).*

Expected Deliverables

1. Providing effective new miticides with different modes of action from currently registered miticides for *Varroa* control to offer more options for Integrated Pest Management and miticides' rotation systems, thereby, reducing the rate of development of resistance.
2. Screening potential alternative antibiotics to currently used fumagillin to improve the *Nosema* treatment.
3. Data to support the registration of potential miticides and antibiotics will be provided to the industry for registration with the Pest Management Regulatory Agency (PMRA) and Health Canada.
4. Providing information to understand current viruses in relation to *Varroa* infestation levels, treatment times, and winterkill to improve recommendations for treatments to protect honey bee health.
5. Improving knowledge of factors that contribute to *Nosema* outbreaks.
6. Developing recommendations to be included in best beekeeping practices to reduce winterkill, reduce the cost of production, and improve beekeeping economics.
7. A comprehensive technology transfer and outreach educational program including on farm demonstrations, newsletter articles, interim reports, and final reports.

4.Research design and methodology

4.1.Continue developing alternative new miticides to enhance the rotation system for sustainable control of resistant *Varroa* mites

The laboratory and field trials for Objective 1 were carried out at the Crop Diversification Center North, Edmonton, Alberta, Canada (53.54 °N, 113.49 °W). All bees used in this bioassay study were sourced from European honey bee (*Apis mellifera*) colonies. The experimental bee colonies were managed using standard Western Canadian management practices.

The activities of 22 formulated products (FPs) and/or active ingredients (AIs) registered for use on plant mites in Canada by the Pest Management Regulatory Agency (PMRA) or in the United States were assessed from 2016 to 2018 for efficacy against *Varroa destructor*, and are listed in Table A4.1. We determined the LD50 (the lethal dose at which 50% of the population is killed in a given period of time) and LC50 (the lethal concentration required to kill 50% of the population) for adult bees and *Varroa* mites for the tested compounds.

Amitraz and associated commercial product, Mitaban® (active ingredient amitraz), were used as positive controls. All FPs, and their AIs were obtained from Sigma-Aldrich or miticide (FP) supply companies. All laboratory tests were performed in a restricted laboratory under the fume hood, according to strict safety practices to avoid any accidental exposure to hazards.

4.1(a) and (b) Laboratory screening to determine activities of tested miticides against *Varroa* mites and safety for honey bees

Laboratory trials:
From 2016 to 2018, 22 active ingredients and formulated products from 18 chemical classes with potential miticide activities were tested (Table A4.1). The miticides used in this experiment were chosen based on their purported target and the inclusion of products across different modes of action. In the laboratory trials, 5, 9, and 14 of the 22 AIs and/or FPs were tested in 2016, 2017 and 2018, respectively. These tests were done to determine contact mite and bee mortality and the lethal concentration (LC50) and dose (LD50).

Testing for Resistance
Mite resistance to Apivar® (active ingredient amitraz) was tested using the Pettis method before the experiments began (Pettis et al. 1998). For this, a group of 150 worker bees with their associated phoretic mites were exposed to a piece of an Apivar® strip (1 X 2.5 cm) in a 500 ml Mason jar for 24 hours at 25°C. After 24 hours the number of dead versus live mites was quantified.

Mite Collection and Brood Preparation
Bees were collected from brood frames sourced from Alberta Agriculture and Forestry (AF) hives with high mite infestation. Once collected, the bees were anesthetized using

carbon dioxide (CO_2) and transferred into a container with a Plexiglas shaker basket. The plastic container was placed on a mechanical shaker and the bees were exposed to CO_2 for five minutes and another five minutes without CO_2. Mites that fell out were collected using a damp fine-tipped paint brush and transferred into a petri dish with a moist paper covering the bottom to prevent desiccation of the live mites (Bahreini and Currie 2015). Capped brood frames were then removed from AF hives with low mite infestations to provide mites with fresh brood to sustain them during the experiment. All attendant bees were removed before the brood frame was placed in a wooden mesh cage and incubated at 33°C. Purple-eyed pupae were carefully removed from cells using forceps and placed on a damp paper towel in a petri dish with a cover and incubated at 33°C until required for experiment.

LD_{50} *Varroa* Mite

A digital micro-applicator fitted with a 10 µl micro-syringe was used to apply candidate compounds. For each compound, across seven concentrations (0%, 1%, 0.1%, 0.01% 0.001%, 0.0001% and 0.00001%) with four replicates each, ten live mites were topically treated with 0.15 µl on each mite' dorsal shield. Treated mites were placed directly into 2 ml polypropylene centrifuge tubes containing purple-eyed pupae for feeding. Tubes were incubated at 33°C for 24 hours. Mite mortality was assessed after 24 hours post-treatment. Control treatments included water, ethanol, acetone and acetonitrile, which were used as solvents. A formamidine (amitraz) was used as a positive control for comparison and 'no treatment' was used as a negative control.

LD50 Honey Bees

Using the same methodology, the micro-applicator fitted with a 25 µl micro-syringe was used to apply each candidate compound. For each compound concentration (0%, 1%, 0.1%, 0.01% 0.001%, 0.0001%, 0.00001, 0.000001 and 0.0000001), each with five replicates, a group of 20 newly emerged worker bees were anesthetized by exposure to CO_2 and each individual bee was topically treated with 1 µl of the candidate compound on the thorax (Fig. A4.1). Once treated, the bees were placed in plastic cages and fed one sugar cube. The bees were incubated at 33°C for 24 hours. Bee mortality was assessed 24 hours post-treatment. Control treatments included water, ethanol, acetone and acetonitrile, which were used as solvents. Again, a formamidine (amitraz) was used as a positive control for comparison and no treatment was used as a negative control.

LC50 *Varroa* Mites

Borosilicate scintillation glass vials (20 ml) were each treated with 0.5 ml of a compound (chemical concentrations: 0%, 1%, 0.1%, 0.01% 0.001%, 0.0001% and 0.00001), with four replicates per concentration per compound, using a pipette. The glass vials were rotated on a cold hot dog roller under a fume hood at room temperature for 2-3 hours until solvents completely evaporated and compounds homogenously coated the inner surface of vials (Fig. A4.2). Ten live mites were placed into the treated vials using a new fine-tipped paint brush. Prepared vials were incubated at 25°C for 6 hours (2016) or at 33°C for 4 hours (2017-2018). Mite mortality was counted 6 (2016) / 4 (2017-2018) hours post-treatment and the surviving mites were transferred into clean scintillation 20 ml glass vials (2016) (Fig. A4.3) or into a clean 2 ml centrifuge tube (2017-2018) containing purple-eyed pupae for feeding. Vials were incubated for an additional 18 hours. Mite mortality was

determined 24 hours post-treatment.

LC50 Honey bees
In 2016 trials, the toxicity of compounds was assessed using a modified Mason jar (500 ml) method (Riusechm 2017) (Fig. A4.4). A piece of plastic strip (2.54 x 11.43 cm) was covered with 0.5 ml of formulated products (at concentrations of: 0%, 0.01%, 0.1%, 1% and 10%). A group of 100-110 newly emerged bees were placed in each prepared Mason jar and given one sugar cube to feed upon. All prepared Mason jars with bees were incubated at 25 °C in the dark. Dead bees were counted in each jar to determine bee mortality after 24 h exposure.

In 2017-2018 trials, glass Mason jars (60 ml) were treated with 0.5 ml of each chemical concentration (0%, 1%, 0.1%, 0.01% 0.001%, 0.0001%, 0.00001, 0.000001 and 0.0000001) with five replicates per concentration per compound, using a pipette. The Mason jars were rotated on a cold hot dog roller under a fume hood at room temperature for 2-3 hours until solvents completely evaporated and compounds homogenously coated the inner surface of jars. One sugar cube was glued to the bottom of the Mason jar before 10 newly emerged bees were added. The top of the jar was covered using a fine mesh screen that was secured using an elastic band (Fig. A4.5). The bees were incubated at 33°C for 24 hours. After 24 hours, post-treatment bee mortality was assessed.

Plastic Cages
To determine bee and mite mortality, approximately 100-120 adult bees from colonies with high mite infestation were placed in 1000ml plastic containers and fed two sugar cubes. Each container contained a plastic strip with an experimental chemical coating. Three doses from each chemical (1%, 0.1%, and 0.01% equal to 5000 µg/strip, 500 µg/strip and 50 µg/strip, respectively) were tested, each with three replicates. After 24 hours of incubation at 33°C, the live and dead mites and dead bees and total bees were counted.

Statistical analyses:
The LC_{50} and LD_{50} values for mite and bee were estimated using the Probit analysis (Finney 1971). The rate of mortality was calculated using the Abbott correction formula for natural mortality in negative controls (Abbott 1925). The variables for cumulative daily mite and bee drop were analyzed using a mixed model ANOVA (PROC MIXED, SAS Institute Inc 2011).

4.1 (c) Semi-field and full scale field testing of promising compounds that demonstrate efficacy against *Varroa* and bee safety in lab trials

Field trials:
2016 Field Trial:
In the fall of 2016, five AIs and/or FPs were tested on single brood chamber (Langstroth) colonies (n=33) fitted with modified screen bottom boards and dead bee traps (Fig. A4.6). Each colony was supplied with 6-8 frames of highly mite infested bees (average 10.24% infestation). The alcohol wash method was used to determine the initial mean abundance of *Varroa* mites from each colony by sampling approximately 300 bees per hive. The bee

samples were washed and the number of *Varroa* mites found per 100 bees represented the initial percentage of infestation. The bee population and brood area in all experimental colonies were visually inspected to estimate the percentage of bees covering each side of the frame.

To minimize genetic variation among treatments, old queens were replaced by new marked queens (Kona, Hawaii). All colonies were randomly assigned into treatments, with three replicates for each treatment. Three additional colonies were treated with Apivar® as a positive control, and three colonies were untreated as a negative control. Ten formulated products (FP) / active ingredients (AI) were tested using a substrate strip (2. 5 x 20 cm). Strips were soaked in a 100 ml dilution (concentrations of: 1%, 5%, 10%, 15% and 20% were tested) for each FP for 24 hours and each concentration (50 mg/strip, 100 mg/strip and 150 mg/strip) of AI was pipetted on strips. Soaked substrate strips were air dried at room temperature under the fume hood. The prepared strips were applied to assigned colonies for each concentration of tested compounds.

To monitor daily mite mortality, a piece of sticky board (30X43 cm) was placed in the tray of the screen bottom board of each hive to collect dying mites that fell through the screen. Sticky boards were removed and replaced with new ones every 1-3 days. Dead mites on sticky boards were counted and mite mortality per day was calculated for each colony. Dead bees were collected from dead bee traps and counted every 24 hours.

2017 & 2018 Field Trials:
In 2017 and 2018, 6 and 3 AIs and/or FPs, respectively, were tested on single brood chamber colonies constructed with three separate compartments. Each compartment contained 3 frames and a total of 24 (2017) or 27 (2018) colonies were tested (Fig. A4.7). The frames and bees in each colony originated from the same mother hive. New Kona queens were introduced to minimize genetic variation and differences in the age of queens. In 2017, five FPs (250 mg/colony, 750 mg/colony and 1000 mg/colony) and one AI (250 mg/colony and 750 mg/colony) were tested on colonies, and three FPs (500 mg/colony, 1000 mg/colony and 1500 mg/colony) were tested on colonies in 2018. In addition, three colonies were left untreated as a negative control in 2017. Strips were treated with chemicals using the same method used in 2016. Treatments were applied to experimental colonies once at the beginning of experiment 2017 and four times (every 7 days) in 2018. To determine daily mite mortality, sticky boards were placed under the hive and changed every 1 – 3 days. Dead mites on sticky traps were counted and mite mortality per day was calculated in each test colony. Mean abundance of mites were determined by sampling and alcohol washing approximately 300 bees per colony before and after of treatments. Apivar® was used as a finishing treatment to quantify the number of *Varroa* mites remaining in the colonies for calculation of treatment efficacy.

Statistical analyses: The variables for cumulative daily mite and bee drop were analyzed using a mixed model ANOVA (PROC MIXED, SAS Institute Inc 2011). This data was also analyzed by a repeated measures analysis of variance using an autoregressive heterogeneous covariance structure. A before and after control impact (BACI) design was used to assess the effects of the treatments on changes of mite levels during experiments. Proportions for mite and bee drop rates were arcsine transformed prior to analyses

(Snedecor and Cochran 1980). Where significant interactions were observed, differences among treatment means were compared using Bonferroni correction (PROC MIXED, SAS Institute Inc 2011)).

4.2. Developing alternative new treatments to enhance the rotation system for sustainable control of resistant *Nosema*

The study was undertaken at the Crop Diversification Centre North (CDCN), Edmonton, Alberta, Canada (53.54 °N, 113.49 °W) in 2017-2018. All bees used in this bioassay study were sourced from European honey bee (*A. mellifera*) colonies. The experimental bee colonies were managed using standard management practices.

4.2 (a) and (b) Laboratory trials:

To prepare the *Nosema* inoculum, mixed-age bees from CDCN colonies were placed in wooden cages (8.5x11.5x15 cm) in the laboratory and fed a 50% sugar solution containing high levels of *Nosema* spores. Each cage was incubated at 33°C for up to 15 days, allowing the *Nosema* spores to reproduce in the intestinal tract of the honey bee (Bahreini and Currie 2015). After 15 days the cages were placed in the freezer to kill all bees. Once dead, the ventriculus was extracted and crushed to release the *Nosema* spores into an aqueous solution. Spores were counted using a microscope and hemocytometer to determine total spores per bee (Cantwell 1970). The viability of extracted spores was quantified via staining procedure using PI (Propidium Iodide), DAPI (4,6-Diamidino-2phenylindole) and fluorescent microscopy (Mcgowan et al. 2016).

Collecting Newly Emerged Bees:

Frames of capped brood were collected from colonies at CDCN with low *Varroa* mite and *Nosema* levels. All bees were removed from the brood frames to avoid contaminating the newly emerging bees, and then the frames were placed in wooden mesh cages (50x26x7 cm). The brood frames were held in an incubator at 33°C until new worker bees emerged from their cells. The bees were then collected in a plastic Rubbermaid® container and exposed to carbon dioxide to anesthetize them. Once anesthetized, 10.0 ±1 grams of bees (100-120 bees) were placed in a Plexiglas® cages (11x12x15 cm) designed specifically for the bioassay experiment (Fig. A5.1). Additionally, a sample of 50 newly emerged bees were collected on day 0 to estimate the pre-experiment *Nosema* level. After two hours in the cage without food, the dead bees were removed and the remaining bees were inoculated with *Nosema* spores using 50% sugar syrup through a 15 mL gravity feeder tube. The inoculation marked the start of the experiment. The cages were placed in the incubator overnight at 33°C. On day 1 of the experiment the dead bees were removed and treatments were applied.

Antimicrobial Application:

Experimental cage bioassays were conducted over 2017-2018. During this time, 11 new antimicrobials were tested in the laboratory, not including fumagillin that was used as a reference treatment. Each treatment had five concentrations with three replicates, with the exception of fumagillin, artemisinin, and mebendazole which had four concentrations and three replicates each in 2018 to test higher concentrations of the antimicrobials (Table

5.1). Positive (fed *Nosema* spores only), negative (fed syrup only), and death (dimethoate 0.0033 mg/ cage; Gough et al. 1994) controls were also included in the experiment.

Each cage was fed *ad libitum* a 50% sugar solution with different concentrations of the candidate antimicrobials over 20 days (Table A5.1). Feeding tubes were monitored and more antimicrobial treatments were added as needed until the completion of the experiment. Each day the dead bees were collected from the cages and placed in centrifuge tubes for subsequent processing. On days 5, 10, and 15, five live bees were collected from each cage. On day 20, all remaining live bees were collected from the cages. Dead bees and live bees from days 0, 5, 10, and 15 were processed in composite samples with a ratio of 1 bee/mL of water to determine the *Nosema* spore abundance (million spores per bee). Day 20 live bees were individually processed with the same ratio to determine *Nosema* spore intensity (million spores per infected-bee), abundance, and infection prevalence (%) (Bush et al. 1997). Once the samples were processed, *Nosema* spores from each experiment were counted using a hemocytometer and microscope (Cantwell 1970).

4.2 (c) Field trials:
A field trial was set up using 18, five-frame Styrofoam nucleus colonies (nucs) (Fig. A5.2). Each nuc had approximately 4-5 frames covered with bees sourced from colonies with low *Varroa* mite and *Nosema* levels. A new mated queen (Kona Queens, Hawaii, USA) was introduced to each colony. Before testing the treatments, the nucs were inoculated with 50% sugar solution containing 6 million *Nosema* spores per mL using a 1 L spray bottle and each nuc was randomly assigned a treatment. Each colony received approximately 10 mL of inoculum by removing each frame and spraying the bees until they appeared wet. All 18 colonies were inoculated using the same solution. Twenty-one days after inoculation when the average spore concentration reached a minimum of 2.5 million spore per bee in all colonies, four concentrations of artemisinin (0.1%, 0.01%, 0.001%, and 0.0001%) and recommended concentration of fumagillin (0.0042%) were tested. Each concentration had 3 replicate nucs, and 3 were left untreated as a control. Each colonies was sprayed with approximately 500 mL treatment solution each time the nucs were treated, based on bee population size, with smaller colonies receiving proportionately less of the treatment. Hives were treated four times from September 21st – Oct 11th 2018, and samples were taken at weekly intervals. Sampled bees were subsequently processed and spores were counted to determine efficacy of chemicals on treating *Nosema* infections.

Statistical analyses:
The cage bioassay experimental design was a split plot treatment arrangement in a randomized complete block design with treatments (candidate compounds, one reference control, one death control, one positive control and one negative control), four or five concentrations for candidate compound and fumagillin treatments, and three replicates for each concentration. For this experiment, candidate compounds and fumagillin treatments were the main plots and concentrations were the sub-plots. The effect of treatments on bee longevity was analyzed using the Kaplan-Meier survivorship analysis (PROC LIFETEST,SAS Institute Inc 2011)). Prior to analyses, variables of bee mortality and Nosema spores were log-transformed. The interaction of treatments and concentrations

on daily bee mortality were analyzed by ANOVA using a repeated measure analysis of variance; in this analysis treatments and concentration were main effects, and day as repeated measure (PROC MIXED,SAS Institute Inc 2011)).

The small colonies (n= 18 nucs) in the 2018 field test were fitted into a completely randomized design with six treatments and three replicates. The changes in *Nosema* spore abundance in treated nucs were analyzed using repeated measure analysis of variance (ANOVA) using an autoregressive heterogeneous covariance structure. Where significant interactions were observed, differences among treatment means were compared using Bonferroni-corrected post-hoc contrasts (SAS Institute Inc 2011).

4.3 Evaluation of factors affecting *Nosema* outbreaks and annual winter mortality

The first objectives of this study were to determine the effect of geographical location, winter management, and fumagillin treatment timing on *Nosema* mean abundance and colony performance and survival in Alberta. The second objective was to determine the seasonal variation and prevalence (the proportion of infected colonies) of *Nosema ceranae* and *Nosema apis* in Alberta

Experiment design
Experiments for both objectives were initiated in May 2017, and will conclude May 2019. Northern and southern Alberta climates were represented by two apiaries near Edmonton, AB and two apiaries near Brooks, AB, respectively. Although the small number of sites may not be adequate to find significant differences between 'north' and 'south', the use of more than one location was necessary to attempt to capture the climatic variation across Alberta. Each apiary contained 32 established double-chamber Langstroth colonies donated by the beekeepers. An additional 8 colonies were located in each apiary specifically for objective 2. Therefore, each apiary had an initial total of 40 colonies. At the beginning of each experiment, all colonies within each apiary were equalized in terms of adult bee and brood populations, and food stores. This was accomplished by collecting all available brood, pollen, and honey frames from the colonies and sharing them equally among the colonies. All bees were shaken into a large screened-cage and distributed equally among the colonies using a 2 L scoop. All colonies were given new marked and mated queens (Kona Queen Hawaii, USA) 1-2 days later. Screened entrances and sugar syrup in in-hive feeders were used to prevent the bees from returning to their original colony for 1-2 days.

Treatments
A split-plot design was used to randomly assign the colonies to the following treatment blocks based upon pre-experiment *Nosema* mean abundance: Spring-Only Treatment, Fall-Only Treatment, Spring and Fall Treatment, Control (no treatment). Each treatment had eight replicate colonies per apiary. The objective 2 colonies were not given any *Nosema* treatment. Therefore, there was a total of 128 colonies for objective 1 and 32 colonies for objective 2 (Fig.1). The formulated product Fumagilin-B® (Medivet, AB, Canada) was used for both spring and fall treatments. To ensure a homogenous mixture, Fumagilin-B® was dissolved in a small amount of water before adding it to the sugar

syrup. The spring treatment used the drenching method which is the preferred method used by Alberta beekeepers in the spring (M. Nasr, former Provincial Apiculturist, personal communication). The treatment consisted of 250 mL of treated 1:1 sugar syrup that was poured onto the bees four times every 4th-day post-treatment. The total amount of syrup applied was 1 liter with a cumulative dose of 120 mg of fumagillin per colony over a 13-day period. The spring treatments were given during June 30-July 18, 2017 and June 13-28, 2018. The fall treatment used the bulk feeding method which is the preferred method used by Alberta beekeepers in the fall (M. Nasr, personal communication) where 3.7 liters of 2:1 sugar syrup containing 120 mg of fumagillin was poured into in-hive feeders. The fall treatments were given September 6 and 8, 2017 and August 29 and 31, 2018. External apiary temperature was collected from nearby weather stations. In 2017-2018, one apiary was wintered outdoors and the other apiary was wintered indoors at each location. In 2018-2019, half of the colonies from each apiary were randomly assigned by treatment to be moved to an indoor wintering facility. The other half remained in the apiary to be wintered outdoors (see Fig. 2).

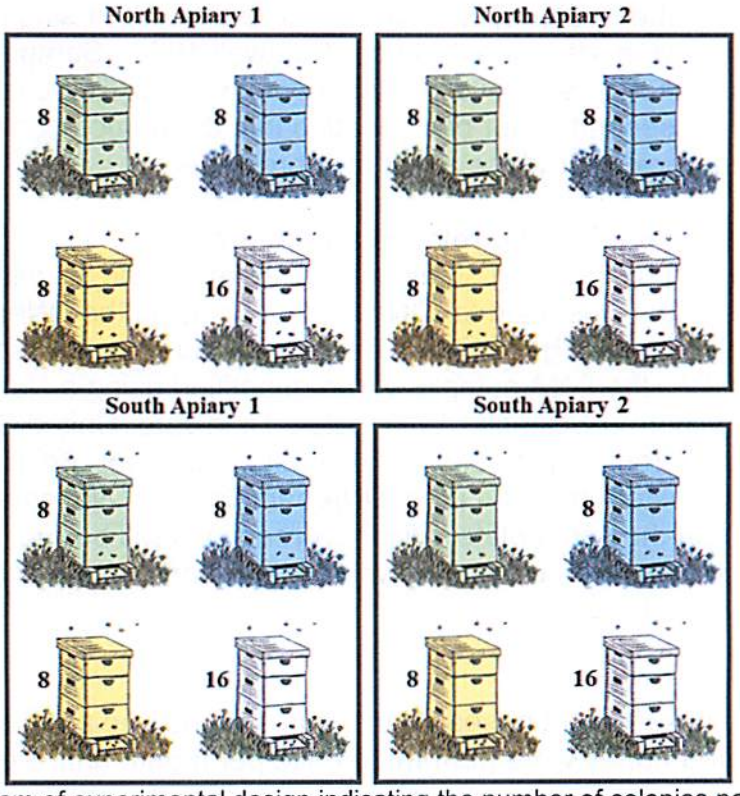

Figure 1. Diagram of experimental design indicating the number of colonies per treatment for all apiaries. Green: Spring-only; Yellow: Fall-only; Blue: Spring and Fall; White: Control + Objective 2

Determining *Varroa* infestation level

In order to isolate the effects of *Nosema* from other variables that could affect bee populations, *Varroa* populations were monitored and controlled when necessary in the experiments. *Varroa* infestation level (mites per 100 bees) were estimated using an alcohol wash (adapted from Gatien and Currie 2003). Samples of approximately 300 bees were collected from the interior brood frames every two weeks (until

colonies were wintered). This was done by sliding the sample jar with 70% alcohol gently down the length of the frame, causing the bees to fall into the jar. Samples were placed on a lab shaker for 10 minutes at 300 rpm. After, the sample was poured into a strainer above a basin and rinsed with a sink sprayer for 1 minute. The number of mites in the basin were recorded. The number of bees in the sample was estimated by dividing the weight of the bees by the average weight of ten bees from three samples. *Varroa* was controlled in all colonies with Apivar® (500 mg amitraz/strip) at the beginning of September both years as some colonies had infestation levels above 3 mites per 100 bees threshold (Nasr and Muirhead 2017).

Determining *Nosema* mean abundance

Nosema samples were collected every two weeks until colonies were prepared for winter. Approximately 100 adult bees were collected from the outer honey frames in the brood chamber or honey supers (Fries et al. 2013) in the same manner as the *Varroa* samples. Samples were prepared by grinding the abdomens of 30 bees with 5 mL of water in a 35 mL conical tissue grinder (VWR). An additional 10 mL of water was used to rinse the tube and grinder. Samples were analyzed using light microscopy and hemocytometer (adapted from Cantwell 1970). Samples were vortexed before analysis to ensure even distribution of spores. The samples were allowed to settle for 30 seconds after being loaded into the hemocytometer. Each sample was counted twice to produce a unit of spores/bee.

Determining *Nosema* species and relative abundance

Samples were prepared by crushing 30 bees in a mortar and pestle with liquid nitrogen.100 µL of sample was used for DNA extraction using the DNeasy Blood and Tissue Kit (QIAGEN, Mississauga, Ontario, Canada) along with its associated protocols. Before qPCR, the DNA sample will be quantified using a NanoDrop Spectrophotometer (Thermo Scientific, Wilmington, Delaware, United States of America). The protocol from Copley *et al.* (2012) will be used for qPCR. Each well will contain 16 µL of MasterMix and 4 µL of DNA sample. Both *Nosema* samples and bee actin will be run on the same plate. All qPCR reactions will be run under the same conditions with standard curves (known serial dilutions of bee actin and *Nosema* spores) and no-template controls.

Evaluating colony strength

The colony strength assessment based on measures of the bee population, total brood area, and stored honey were performed once per month until the colonies were wintered. Bee population was estimated by recording the approximate percentage (to the nearest quarter) of bees covering each side of the hive's frames. It was assumed that 2430 bees fully cover both sides of a frame (Burgett and Burikam 1985). Frames containing brood were photographed and analyzed using ImageJ (National Institute of Health, USA) to calculate the square centimeters of brood per colony. Colonies with brood diseases and other pests (excluding *Nosema*) below the economic thresholds were considered healthy. The amount of stored honey was estimated by recording the approximate percentage (to the nearest quarter) of capped honey on each side of the hive's frame. Queen presence, supersedure, and acceptance, as well as colony mortality and viability, were

recorded. Colony mortality was defined as no live queen or bees in the hive. Colonies were considered non-viable from a commercial standpoint when there were less than 4 frames of bees in the colony in early spring or late fall.

Post-winter evaluation

Colonies will be sampled and evaluated when indoor colonies are moved out and outdoor colonies are unwrapped in the spring of the following year. Both *Nosema* and *Varroa* samples will be taken. If there are no live bees within a colony, a *Nosema* sample will be taken using the dead bees from the bottom board. Cluster size will be evaluated initially (Nasr et al. 1990) as early spring weather may be too cold for an extensive colony assessment. From this, colony mortality and viability will be determined. A colony strength assessment of bee population, total brood area, and stored honey will be done on all colonies. If the colony has no live bees, an attempt will be made to deduce the cause of colony death.

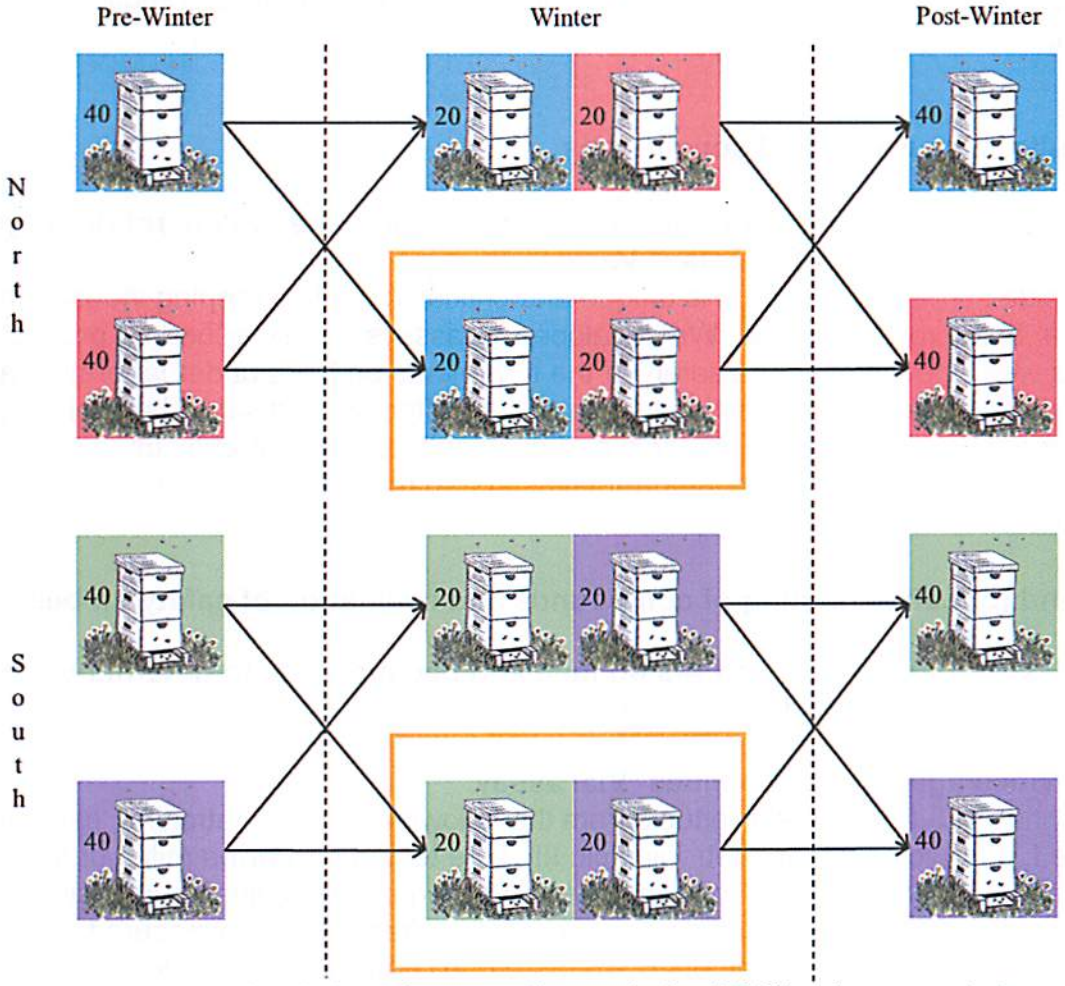

Figure 2. Diagram of the before-after-control-impact design (BACI) and movement of colonies for winter at each location for 2018-19. The number next to hives indicates the number of colonies (which were randomly assigned by treatment block). Colored hive images represent the four apiaries. A yellow square indicates an indoor wintering facility.

Statistical analysis

It is acknowledged that there is some spatial pseudoreplication in these experiments due

to practical considerations related to the need to winter colonies in buildings. To combat this, a before-after-control-impact (BACI) design (Smith 2002) will be employed to compare variables by looking at the relative changes in response to before and after wintering (Fig. 2). As the same colonies will be observed several times, a repeated measures generalized linear mixed-model (GLMM) will be performed with the following variables:

- Determine the effect of location and wintering method on *Nosema* mean abundance, species prevalence, and colony health (brood area, bee population, and amount of honey)
- Determine the effect of fumagillin treatment on *Nosema* mean abundance, species prevalence, and colony health (brood area, bee population, and amount of honey).
- Determine the variation of *Nosema* mean abundance over time (seasonality)

Any necessary data transformations will be performed to meet the assumptions of a GLMM prior to analysis.

5. Results, discussion and conclusions

5.1. Continue developing alternative new miticides to enhance the rotation system for sustainable control of resistant *Varroa*

This miticide screening research is unique and critical to the beekeeping industry, not only in Alberta, but across the world. We developed bioassays and screened 22 products on mites, as well as evaluating the safety of the treatments on bees under laboratory and field conditions. Our work identified two potentially effective and safe AIs belonging to two different chemical groups (pyrazole and quinazoline) from currently used (non-apicultural) miticides. This is an important first step to make more products available to beekeepers for *Varroa* control.

5.1 (a) and (b) Lab screening of compounds and evaluation of safety on bees

2016 - Assessment of FPs and AIs on mite and bee mortality to determine the LC_{50} under laboratory and field conditions

FPs activities against *Varroa* mites- Vial assay:

The activities of 5 formulated products from the following classes: tetrazine, carbazate, quinoline I, tetronic acid I, and tetronic acid III, were tested on *Varroa* mites under laboratory conditions in 2016. Formamidine (active ingredient amitraz) was used as a positive control. There was significant mite mortality after a 6 hour exposure to FPs using the glass vial method ($F= 11.36$; $df= 6, 18$; $p< 0.0001$). Similar results were reported after 24 hours ($F=36.85$; $df= 6, 118$; $p<0.0001$) (Fig. A4.8). Formamidine had the highest mite mortality of all treatments. Tetrazine, carbazate, quinoline, I and tetronic acid I had similar mite mortalities to one another, but lower than that of formamidine (amitraz) and higher than tetronic acid III. Partitioning the interaction of the FPs treatment and concentrations within each tested product indicated significant differences in 24 h mite mortality within tetrazine, carbazate, tetronic acid III, quinoline I, and tetronic acid I. The contact toxicity

(LC_{50}) of the five FPs to mites were calculated (Table A4.2).

AI activities against *Varroa* mites- Vial assay:
When the AIs of these FPs were assessed for miticidal activities against *Varroa* mites using the vial test, there were significant differences in mite mortalities after 6 and 24 h exposures (Fig. A4.9). Formamidine (amitraz) and carbazate had the highest *Varroa* mortality. Partitioning the interaction of treatment and concentration within the treatment indicated significant differences in 24 h mite mortality only within carbazate and tetronic acid III. The contact toxicity (LC_{50}) of the AIs to *Varroa* mites after 24 h is presented in Table A4.2.

FPs activities against bees- Mason Jars (500 mL):
There were significant differences between the rates of bee mortality across formulated products when tested in large Mason jars ($F=58.00$; df= 6, 100; $p<0.0001$). The doses of formamidine (amitraz) 1% and 10% caused bee mortality at rates of 37% and 96%, respectively. Post-hoc analyses excluding formamidine failed to identify differences among the other FPs, and there were no significance differences among the treatments and control ($F=2.22$; df= 5, 84; $p=0.0598$).

AI activities against bees- Mason Jars (500 mL):
Our results demonstrated that formamidine (amitraz) had the highest bee mortality rate after 24 h exposure. When formamidine was excluded from the analyses due to high bee mortality, carbazate had the highest bee mortality, and the rates of bee mortality in quinoline and tetronic acid II were similar to the control group.

Field assessment of FPs *Varroa* mites and honey bees:
The daily mite mortality varied significantly for FPs of quinoline, tetrazine, tetronic acid I and formamidine tested in the field. High daily mite mortality was observed for both Apivar® and its AI formamidine (amitraz), and both were significantly higher than quinoline, tetrazine, tetronic acid I and control ($F=12.47$; df= 6, 92; $p<0.0001$). Mean daily bee mortality was not significantly different among tested FPs ($F=0.84$; df= 6, 92; $p=0.5428$) (Table A4.3).

Field assessment of AIs on *Varroa* mites and honey bees:
The rate of mite mortality for AIs from classes: quinoline, carbazate, tetrazine, tetronic acid I, tetronic acid III, and the control were not significantly different from one another ($F= 2.80$; df= 5, 14; $p= 0.0591$). When AIs were tested in the field in 2016, the mite mortality was similar among AIs, but tetronic acid I had a slightly higher mite mortality than other tested compounds. Overall average daily bee mortality was not significantly different among treatments ($F=1.71$; df= 5, 14; $p=0.1979$). Further testing showed that higher daily bee mortality was found in 3% formamidine (amitraz) and high concentrations (10%) of tetrazine, tetronic acid I and quinoline I (Treatment*concentration: $F= 4.76$; df= 12, 56; $p<0.0001$) (Table A4.3).

2017 - Assessment of FPs and AIs on mite and bee mortality and determining the LC_{50} and LD_{50} under laboratory and field conditions
When used as a positive control to verify the results of the experiment, dimethoate

resulted in 100% bee mortality. Solvents were used as negative controls and did not have any significant effects on bee mortality.

FPs/AIs activities against *Varroa* mites- Vial assay:
When FPs were tested on *Varroa* using the glass vial test, daily mite mortality was variable for the tested products ($F= 14.76$; $df= 9, 230$; $p<0.0001$). When mites were treated with AIs, significant differences in mortality were observed. The highest mortalities were recorded in mites exposed to formamidine (amitraz) and pyrazole I after 4 hours of treatment ($F= 20.82$; $df= 9, 230$; $p<0.0001$) (Fig. A4.10) and 24 hour tests ($F= 11.93$; $df= 9, 230$; $p<0.0001$) (Fig. A4.11). However, during the 24 h test, formamidine, avermectin I, pyrethroid II, pyrrole, pyrazole I and tetronic acid II had significantly higher mite mortalities than the other AIs ($F= 11.93$; $df= 9, 230$; $p<0.0001$) (Fig. A4.11), but were not significantly different from each other. The LC_{50} values for tested products are presented in Table A4.2.

FPs/AIs activities against *Varroa* mites- Micro-applicator assay:
Using the micro-applicator to apply specific doses to mites, formulated products differed in their efficacy against *Varroa* after 24 h exposure ($F= 47.77$; $df= 9, 262$; $p<0.0001$; Fig. A4.12). Among tested compounds, higher mite mortality was observed when mites were topically treated with formamidine, pyrethroid II, pyrrole and pyrazole I compared to avermectin I, quinoline I, tetrazine, benzoylacetonitrile, tetronic acid I and tetronic acid II. The LD_{50} values for tested products are presented in Table A4.4.

FPs/AIs activities against honey bee - Mason jar assay (60 mL):
Formulated products avermectin I and formamidine (amitraz) resulted in significantly higher bee mortality than the other 8 tested products ($F= 59.79$; $df= 9, 467$; $p<0.0001$) (Fig. A4.13).

Bee mortality also varied significantly among the tested AI compounds after 24 h exposure ($F 26.25=$; $df= 9, 379$; $p<0.0001$) (Fig. A4.14), with pyrethroid II, tetronic acid I and tetronic acid II causing higher 24h bee mortality than controls. Although these compounds resulted in high bee mortality, they were not significantly different from one another. The LC_{50} values for tested products are presented in Table A4.2.

FPs/AIs activities against honey bee - Micro-applicator assay:
The formulated products avermectin I, pyrethroid II and benzoylacetonitrile killed more topically treated bees than did formamidine (amitraz) after 24h ($F= 10.29$; $df= 9, 381$; $p<0.0001$) (Fig. A4.15).

For AIs tested with the micro-applicator assay, more bees were killed during a 24h-period when they were topically treated with avermectin I, pyrethroid II and pyrrole than formamidine (amitraz) ($F= 27.22$; $df= 9, 384$; $p<0.0001$) (Fig. A4.16). The LD_{50} values for tested products are presented in Table A4.4.

2018 - Assessment of FPs and AIs on mite and bee mortality and determining the LC_{50} and LD_{50} under laboratory and field conditions

AI activities against *Varroa* mites-Vial assay:
When AIs were tested using the glass vial test in 2018, mite mortality was significantly lower for the oxazoline and thiazolidinone treatments after 4h than other tested AIs. Quinazoline resulted in the higher mite mortality 4h post-treatment compared to formamidine ($F= 15.02$; $df= 9, 212$; $p<0.0001$) (Fig. A4.17). Pyrazole I, organochlorine I, quinazoline, pyrazole II and pyrazole III resulted in significantly greater mite mortality than formamidine 24h post-treatment, but were not significantly different from one another ($F= 28.3$; $df= 9, 211$; $p<0.0001$) (Fig. A4.18). The LC_{50} values for tested products are presented in Table A4.2.

AI activities against *Varroa* mites- Micro-applicator assay:
Using the micro-applicator to apply specific doses, the mite mortality varied among tested AIs ($F= 25.43$; $df= 9, 205$; $p<0.0001$) after 24 h exposure (Fig. A4.19), with high mite mortality observed when mites were topically treated with pyrazole I, pyrazole II, or pyrazole III. The LD_{50} values for tested products are presented in Table A4.4.

AI activities against honey bee - Micro-applicator assay:
After 24h, pyrazole II, pyrazole III and pyrethroid I killed more topically treated bees than formamidine (amitraz); fewer honey bees were killed during a 24h-period when they were topically treated with oxazoline and thiazolidinone, similar to control treatment bee mortality. ($F= 8.29$; $df= 9, 210$; $p<0.0001$) (Fig. A4.20). The LD_{50} values for tested products are presented in Table A4.4.

AI activities against honey bee - Mason jar assay (60 mL):
Mortality of the bees varied significantly among AIs over the 24 h testing period in the Mason jar assay ($F= 7.77$; $df= 9, 210$; $p<0.0001$) (Fig. A4.21). Pyrazole II caused significantly higher bee mortality than organochlorine I, pyrazole III and pyrethroid I, which in turn resulted in higher bee mortality than formamidine. When compared to formamidine (amitraz), pyrazole I, oxazoline, thiazolidinone, quinazoline and pyrazole III were safer for bees. The LC_{50} values for tested products are presented in Table A4.2.

Evaluation of Laboratory Assessments for mite and bee mortality using Plastic cages:
Mite and bee mortality was assessed when *Varroa*-infested worker bees were exposed to plastic strips covered with AIs. Results suggest higher bee mortality for Pyrethroid II, Pyrrole, Avermectin I, Pyrethroid I, and Organochlorine I ($F= 25.24$; $df= 22, 178$; $p<0.0001$) (Fig. A4.22). Meanwhile, greater mite drop was observed for Formamidine (amitraz), Pyrethroid II, Avermectin II, Quinazoline, Pyrethroid I, Pyrazole I and Organochlorine I ($F= 11.31$; $df= 22,178$; $p<0.0001$) (Fig. A4.23) (Table 4). Overall, this bioassay test suggests that Quinazoline and Pyrazole I had relatively higher efficacy in mite control and were safe for bees.

Collectively, our laboratory research from 2016 to 2018 for screening miticides with different modes of actions is unique and imperative to the beekeeping industry, not only in Alberta, but across the world. We have established bioassays for screening the efficacy of compounds on mites and their safety to bees under laboratory conditions. Overall, this technology was used to screen 11 FPs and 22 AIs from 18 chemical classes for efficacy

against Varroa mites and safety to honey bees. The laboratory trials identified 4 potential AIs that belong to 4 unique acaricides/insecticides groups (Benzoylacetonitrile, Pyrazole, Tetronic acid and Quinazoline) that are different from currently used groups. In the next step, these were tested under semi-field conditions.

5.1 (c) Semi-field and full scale field testing of promising compounds that demonstrate efficacy against *Varroa* and bee safety in lab trials

Field assessment of FPs and AIs on *Varroa* mites and honey bees 2017
In the fall of 2017, the FPs avermectin I, pyrrole, benzoylacetonitrile, tetronic acid II, pyrazole I, and tetronic acid I were tested in the field, in addition to the AI tetronic acid I. Avermectin I (750 mg/colony) killed the entire bee population in the treated colonies and was removed from subsequent data analyses (Table A4.5). Overall, a significantly higher daily mite drop was observed for all treatments when compared to control ($F= 6.02$; $df= 6, 29$; $p= 0.0003$). Pyrazole I and tetronic acid II caused the highest mite mortalities. Analysis of variance on treatment*dose interaction showed significant interactions, meaning that the effect of each treatment depended on the dose ($F= 11.27$; $df= 11, 24$; $p<0.0001$). The order of daily mite mortality resulting from each treatment, from highest to lowest, was pyrazole I 750 mg/colony > pyrazole I 250 mg/colony> tetronic acid I 750 mg/colony>tetronic acid II 750 mg/colony> benzoylacetonitrile 750 mg/colony> tetronic acid I 750 mg/colony. The treatment of infested bee colonies with pyrazole I 750 mg/colony, benzoylacetonitrile 1000 mg/colony and tetronic acid II 750 mg/colony decreased the mite level by 89%, 67% and 26%, respectively, during treatment period (Fig. A4.24).

Field assessment of FPs and AIs on *Varroa* mites and honey bees 2018
In the fall of 2018, the products benzoylacetonitrile, tetronic acid II, and pyrazole I were tested in the field to determine their efficacy against *Varroa* in honey bee colonies. Overall, the cumulative daily mite mortality rate for pyrazole I and tetronic acid III was significantly higher than benzoylacetonitrile ($F= 6.24$; $df= 2, 24$; $p= 0.0066$; mean mite population accounting for starting mite population) (Fig. A4.25). Repeated measure analyses showed that the cumulative daily mite drop was highest when colonies were exposed to pyrazole I, followed by tetronic acid II and then benzoylacetonitrile ($F= 19.85$; $df= 47, 358$; $p< 0.0001$) (Fig. A4.26). However, there was a significant interaction between dose and treatment ($F= 4.45$; $df= 8, 18$; $p= 0.0041$) (Fig. A4.27), but cumulative daily mite mortality was not dose-dependent. Daily mite mortality during the trial was significantly greater for pyrazole I compared to tetronic acid II and benzoylacetonitrile ($F= 19.85$; $df= 47, 358$; $p< 0.0001$) (Fig. A4.28) and increased after each treatment. Daily mite mortality was dose-dependent for pyrazole I, with higher mite mortality observed over the treatment period when higher doses were applied ($F= 9.08$; $df= 143, 262$; $p< 0.0001$) (Fig. A4.29).

5.2. Developing alternative new treatments to enhance the rotation system for sustainable control of resistant *Nosema*

The research we conducted in 2017 demonstrated that artemisinin is a potential effective antibiotic for *Nosema* treatment. The products from the 2018 screening program are still

under investigation. Although the efficacy of artemisinin is lower than fumagillin, bee survivorship is significantly higher in treated bees under laboratory conditions. Similar to fumagillin, artemisinin could prevent development of *Nosema* spores in treated bees similar to fumagillin when bees are continually exposed to the compound under field conditions.

5.2 (a) and (b) Lab screening of 10 compounds and evaluation of safety on bees
A list of all tested compounds is in Table A5.1.

2017 – Lab tests for efficacy against *Nosema*
Nosema infection prevalence, intensity, and abundance were similar in all inoculated samples before testing the candidate antibiotics (day 0). The negative control, fumagillin, artemisinin, and mebendazole had a significantly lower intensity ($F= 52.2$; $df= 8, 3263$; $P< 0.0001$) and abundance ($F= 92.73$; $df= 8, 4074$; $P< 0.0001$) of *Nosema* spores than did the positive control, followed by amprolium and quinine (Fig. A5.3) in live bees 20 days post-inoculation. The abundance of *Nosema* spores in dead bees was lower in the negative control, dimethoate, and fumagillin treated bees than other products ($F= 27.29$; $df= 9, 102$; $P<0.0001$) (Fig. A5.4). However, the abundance of *Nosema* spores in live bees collected at days 0, 5, 10 and 15 post- inoculation increased after day 5, and the highest abundance was recorded in the genestin, positive control, and metronidazole treatments ($F= 22.35$; $df= 8, 102$; $P<0.0001$) (Fig. A5.5). A significantly lower rate of *Nosema* prevalence was observed in the negative control, followed by quinine, artemisinin, mebendazole and fumagillin treatments ($F= 8.93$; $df= 8, 87$; $P<0.0001$) (Fig. A5.6).

All caged-bees were killed by day 7 when fed dimethoate as death control. Daily bee mortality was found to be significantly less for artemisinin and mebendazole when compared to the negative control treatments ($F= 9.78$; $df= 9, 105$; $P< 0.0001$) (Fig. A5.7). The Kaplan-Meier survival analysis showed significant differences among treatments. Artemisinin, mebendazole, and the negative controls showed higher bee survivorship, with more than 70% of the bees alive 20 days post-inoculation compared to 30% bee survival in the fumagillin treatment (Long-Rank: $X^2= 7414.6054$; $df= 9$; $P < 0.0001$; Wilcoxon: $X^2= 7373.8263$; $df= 9$; $P < 0.0001$) (Fig. A5.8).

2018 – Lab tests for efficacy against *Nosema*
Similar daily bee mortality was observed in the artemisinin, mebendazole, and fumagillin treatments when bees were exposed to high doses of antimicrobials (> 0.1%), but lower than the bee mortality observed in the positive control ($F= 3.53$; $df= 4, 37$; $P = 0.0155$) (Fig. A5.9). Higher doses of artemisinin (0.5% and 1%) cause slightly higher bee mortality compared to mebendazole and fumagillin ($F= 1.15$; $df= 13, 28$; $P= 0.3595$). The survival rate of bees dropped to less than 70% when bees were exposed to higher doses of fumagillin, mebendazole and artemisinin for 20 days (Long-Rank: $X^2= 264.6489$; $df= 4$; $P < 0.0001$; Wilcoxon: $X^2= 289.2176$; $df= 4$; $P < 0.0001$) (Fig. A5.10). Spore counting and analysis of prevalence, abundance and intensity of *Nosema* spores from 2018 are in process.

Bees treated with curcumin, fenbendazole, ornidazole and nitazoxanide

showed similar daily bee mortality to the negative control (F= 2.96; df= 6, 74; P = 0.0121) (Fig. A5.11). More than 70% survival rate was observed for nitazoxanide, negative control, fenbendazole, and ornidazole treatments (Long-Rank: X^2= 634.389; df= 6; P < 0.0001; Wilcoxon: X^2= 545.909; df= 6; P < 0.0001) (Fig. A5.12). Spore counting and analysis of prevalence, abundance and intensity of *Nosema* spores from 2018 experiments are in process.

5.2 (c) Semi-field and full scale field testing of promising compounds that demonstrate efficacy against *Nosema* and bee safety in lab trial

2018 Field tests

Nosema-infested nucs were treated with artemisinin and fumagillin. Repeated-measures ANOVA indicated a significant difference in *Nosema* infestation level over time (F= 10.92; df= 26, 113; P< 0.0001) (Fig. A5.13). The level of *Nosema* was similar between artemisinin and fumagillin during treatment times (periods 1 and 6), however, *Nosema* spores significantly increased in artemisinin-treated colonies two weeks after the last treatment, meanwhile, spore abundance increased after three weeks for fumagillin (F= 5.78; df= 53, 86; P< 0.0001).

5.3. Evaluation of factors affecting *Nosema* outbreaks and annual winter mortality – Punko thesis

Preliminary Results 2017-2018

Nosema and *Varroa* samples were taken seven times from June to September 2017, for a total of 960 *Nosema* samples and 960 *Varroa* samples. Colony strength assessments were done four times: in July, August, and two in September. Also, over 8000 photos of brood were taken. This report presents the results of preliminary data for the bee population, brood area, and *Nosema* and *Varroa* samples.

The effect of spring treatment, location, and sampling date on *Varroa* infestation was analyzed with PROC MIXED (SAS v.9.4, SAS Institute 2019) using a repeated measures design with hives as the subject and sampling date as the repeated measure using the REML statement (restricted maximum likelihood). *Varroa* infestation was arcsin transformed to meet the assumption of normality. A significant interaction for location*date (F=6.06, df=6, 706, P<0.0001) was partitioned using the SLICE option in the LSMEANS statement. There was no effect of spring treatment with fumagillin on mite infestation (F=1.55, df=1,124, P=0.2152). Mite infestation levels were significantly higher overall in the southern apiaries than the northern apiaries (Fig. A6.1). The average *Varroa* mite mean abundance by location stayed below 1% (1 mite per 100 bees) over the entire experiment. However, there were individual colonies in all apiaries, except N2, that were above the economic threshold of 3%, the highest being at 9.1% infestation immediately before treatment. The treatment was effective at reducing the average mite infection by location to below 0.5%, with the highest colony infection at 2.0% after treatment.

The effect of spring treatment, apiary, and sampling date on Nosema mean abundance was analyzed with PROC MIXED (SAS) using a repeated measures design with hives as

the subject and sampling date as the repeated measure using the REML statement. *Nosema* infection was logarithmically transformed to meet the assumption of normality. The interactions apiary*date and spr*date were found to be significant ($F=6.93$, df=9, 349, $P<0.0001$ and $F=2.75$, df=3, 349, $P=0.0428$, respectively). N1 had significantly higher *Nosema* infection than the other apiaries at the beginning of the experiment (Fig. A6.2). Following spring treatment, N2 had significantly higher *Nosema* mean abundance than the other apiaries. At the end of July, northern colonies had a significantly higher infection than the southern colonies. Again, N2 had a higher infection than the other apiaries, except for S2. For spr*date, the interaction was partitioned by date using the SLICE option in the LSMEANS statement. Before treatment, treated and not-treated colonies had the same level of *Nosema* infection (Fig. A6.3). Following spring treatment, treated colonies had significantly less *Nosema* mean abundance than not-treated colonies. However, subsequent samplings showed no significant differences between colonies that did or did not receive spring treatment. Overall, *Nosema* infection decreased as summer progressed.

The effect of spring treatment, location, and sampling date on brood area was analyzed with PROC MIXED (SAS) using a repeated measures design with hives as the subject and month as the repeated measure using the REML statement. Brood area was square root transformed to meet the assumption of normality. The main effects location and date were significant ($F=46.02$, df=1, 349, $P<0.0001$ and $F=101.67$, df=3, 124, $P<0.0001$, respectively). Spring treatment had no effect on brood area ($F=0.06$, df=1, 124, $P=0.8105$). The northern apiaries had significantly greater brood area than the southern apiaries at all sampling dates (Figure A6.4). Colonies began the experiment with approximately 2600 to 3800 cm^2 of brood. At the last sampling date, colonies had approximately 570 to 1100 cm^2 of brood.

The effect of spring treatment, apiary, and sampling date on bee population was analyzed with PROC MIXED (SAS) using a repeated measures design with hives as the subject and month as the repeated measure using the REML statement. Bee population was square root transformed to meet the assumption of normality. A significant interaction for date*spr*apiary was observed ($F=2.33$, df=9, 334, $P=0.0148$). The interaction was partitioned by round*yard using the SLICE option in the LSMEANS statement. The only significant differences were found in the N1 apiary before and immediately following treatment (Figure A6.5). Colonies began the experiment with between 10, 000 and 19, 000 bees or 4-8 frames. When they were last checked at the end of September before going into winter, colonies had approximately 20, 000 bees or 8 frames.

After winter, colonies were evaluated for mortality. Apiaries N1 and S1 were wintered outdoors whereas N2 and S2 were wintered indoors. During the experiment, some colonies died, resulting in less than 40 colonies going into winter (Table A6.1). Therefore, winter mortality was calculated by dividing the number of dead colonies after winter by the number of live colonies that were overwintered. The south had slightly higher winter mortality than the north (Table A6.2). Outdoor-wintered colonies had double the winter mortality of indoor-wintered colonies (Table A6.2), with S1 having the highest winter mortality of 34.3% (Table A6.1). The indoor-wintered apiaries had the same winter mortality, whereas the outdoor-wintered apiaries winter mortality was higher in the south

than the north (Table A6.1). Colonies were also evaluated for viability after winter. Colonies were given a cluster score between 0 and 5 based on the number of frames of bees. A cluster score below 3 was considered to be not viable. S1 and N2 had the highest number of non-viable colonies (Table A6.1). The south colonies had more non-viable colonies than the north colonies. Indoor- and outdoor- wintered colonies had about the same number of non-viable colonies.

When broken down by treatment, slightly more colonies that were treated in the spring and spring and fall died compared to the control (Table A6.3). In the north, mortality was higher for fall only and spring/fall treatment colonies when compared to control. In the south, only the fall-treated colonies had less winter mortality than the control. There was no difference between treatments for outdoor-wintered colonies. Spring/fall treated colonies had the highest mortality for indoor-wintered colonies when compared to the control. As for the number of non-viable colonies, the fall treatment had the most overall (Table A6.4). There was no difference between treatments for the north. For the south and outdoor-wintered, more non-viable colonies occurred in those treated in the fall compared to the control. Spring-only and spring/fall treatment colonies had less non-viable colonies than the control when wintered indoors.

There were significant differences in *Nosema* mean abundance among the apiaries at the first four sampling dates. At least one of the north apiaries always had the highest *Nosema* mean abundance which indicates that there are likely differences between the north and south locations that result in differences in the progression of nosemosis. Furthermore, colonies that were treated with fumagillin had significantly lower *Nosema* abundance shortly after treatment, demonstrating the effectiveness of the spring treatment, at least in a short time frame. However, *Nosema* mean abundance continued to naturally decrease with each following sample date and there were no significant differences at subsequent dates. In the spring, colonies are rapidly building up their population for the honey flow which peaks in July - August. Therefore, *Nosema* prevalence likely decreases concurrently with the increasing population. Since mean abundance is a combination of intensity and prevalence, it is affected by this change. Differences in *Nosema* infection may be seen again when the population decreases going into winter.

Although apiaries at the northern sites had higher *Nosema* abundance, they also had greater brood area than the south apiaries. There was no effect of spring treatment on brood area which shows that treatment does not negatively (or positively) affect this aspect of the colony dynamics. In north apiary 1, treated colonies had more individual bees than untreated colonies both before *and immediately after treatment*. The goal of equalizing the bees and brood among all the colonies was that they would have equal amounts of bees, brood, and *Nosema* infection. However, it appears that this was not successful in north apiary 1 and may have contributed to the significant difference in bee population at the second sampling date.

The preliminary winter mortality and viability data suggests that there are differences among the locations, wintering methods, and treatments. Winter mortality appears to be more affected by wintering method and viability more affected by location. Furthermore,

the winter mortality of outdoor-wintered colonies seems to vary with location. There were differences in winter mortality between treatments which varied with location and wintering method. The fall treatment had opposite effects on mortality depending on location. There were also differences in viability between treatments depending on location and wintering method. Acknowledging both mortality and viability can show treatments that may result in low mortality but high non-viability, such as with the fall treatment in the south. Statistical testing is required to determine the significance of these results.

6. Discussion, implications and value of developed knowledge:

One of the greatest threats to the honey bee worldwide is the parasitic mite, *Varroa destructor*. The life cycle of the *Varroa* mite is tightly adapted to the development of the honey bees. Several studies have documented the ill effects of *Varroa* infestation on bees including reduced lifespan, decreased survivorship and weight loss in drones (reviewed by Rosenkranz et al. 2010). *Varroa* mites are also efficient vectors for transmission of viral diseases (Francis et al. 2013). Given the complex interactions between honey bees and *Varroa* mites, synthetic miticides play a major role in the management of *Varroa* mites. Most beekeepers incorporated synthetic miticides into their management plans. Within a few years of repeated use, beekeepers began to report that products like Apistan® and Checkmite+® were no longer effective for *Varroa* mite control. In recent years, beekeepers in multiple regions have reported Apivar® resistance developing in their operations.

Our reported research for screening miticides with different modes of actions is unique and imperative to the beekeeping industry, not only in Alberta, but across the world. We have established bioassays for screening the efficacy of compounds on mites and their safety to bees under laboratory and field conditions. This technology was used to screen 22 FPs and their AIs for efficacy against *Varroa* mites and safety to honey bees. Our work identified 2 potential AIs that belonging to 2 unique acaricides/insecticides groups that are different from currently used groups. This is an important first step to make more products available to beekeepers for *Varroa* control, but further development must be supported by the FP registrants. These products would be the first addition of synthetic products for *Varroa* control since the early 1980's.

Another miticide was developed in our research project, HopGuard II. Early studies at our laboratory showed that HopGuard™ has limited efficacy (less than 45%) under Alberta conditions when applied to honey bee colonies (Vandervalk et al. 2014).Our data, supported by observations in the field, showed that the causes of low efficacy included: the presence of brood in treated colonies, the limited period of bees' exposure to applied HopGuard, and bees chewing and removing much of the applied strips within few days of treatment Our results led also to the discovery that beta acid, the active ingredient in HopGuard works as a contact miticide. All of these findings helped us to develop a new strip with a proper dose of active ingredient to control *Varroa*. The efficacy of HopGuard II increased to up to 95% and continued to be safe for use in bee colonies. HopGuard II has now proven to be an effective tool for *Varroa* control in an integrated pest management system applied by beekeepers. This new product is currently under review by the PMRA

for registration to be used in Canada. It should be available for beekeepers in 2019. This research has added a new natural product extracted from hops for use in *Varroa* control. It is considered 'Generally Recognized As Safe' (GRAS) for human consumption according to the United States Food and Drug Administration Agency (US-FDA). Thus, it would be also a useful tool in organic production systems.

Our evaluation of oxalic acid applied in cardboard strips and Scott® blue shop towels as described by Maggi et al. (2015) and Oliver (2017 a, b, and c) under Alberta conditions showed that these methods have potential to control mites in the fall when minimal brood or no brood is found in bee colonies. In our testing the cardboard strips provided higher efficacy (96.8%) with minimal variations among tested colonies in comparison to the Scott® blue shop towels (85.6%). Further research is required to investigate the side effects on honey bees through the season and the potential for residues in honey currently unknown. Once these data are collected, there is potential for these two methods to be registered by PMRA and recommended for use by beekeepers. Thus, these methods could become an inexpensive and safe method of applying oxalic acid in bee colonies and be integrated to be another component of the integrated pest management system for *Varroa* mite control in Alberta.

We further identified a compound, artemisinin as a potential effective antibiotic for *Nosema* treatment, and provided data on its relative safety for honey bees.

These established new bioassays can be used in the future not only for developing miticides and antibiotics, but also to study toxicity of any agrochemicals to bees. Thus, this research will help in the advancement of agricultural science. The discovery of new types of chemicals that can be used as miticides or antibiotics will likely play a major role in beekeeping in the future. This work helps beekeepers enhance their ability to control *Varroa* mite populations, treat *Nosema*, and keep healthy bees. This can lead to substantial economic benefits for the beekeeper and farmers. Including these new treatment options in Integrated Pest Management (IPM) systems is vital for keeping healthy bees and thriving industry

Section D: Benefits to the industry

The Alberta apiculture industry accounts for over 40% of Canadian colonies and honey production (Mukezangango and Page 2017). It also produces other commodities such as beeswax, pollen, queens, and nucleus colonies. The most economically valuable service provided by Alberta beekeepers is pollination. Pedigreed hybrid seed canola production in southern Alberta is a multi-million dollar industry that relies on bee pollination (Clay 2009, Mukezangango and Page 2017). In addition, some beekeepers move colonies to British Columbia to pollinate blueberries and other fruit crops in the spring (Laate 2017). Therefore, it is imperative to have sustainable production of healthy honey bee colonies. The Alberta Beekeepers Commission has identified honey bee health as the most challenging problem facing the beekeeping industry. The development of new control agents to enhance the sustainability of the integrated pest management (IPM) system for

Varroa mites and *Nosema* answers these challenges. The outcomes of this project have provided the following results:

1) Identified 2 potential new miticide classes (Pyrazole and Quinazoline) with unique modes of action that could be integrated into the integrated pest management system for *Varroa* mites.
2) Improved HopGuard application methods to produce an effective mitcide to be registered in Canada for beekeepers' use for *Varroa* control. **HopGuard II** is a bio-pesticide miticide formulated as a strip impregnated with potassium salts of Hop beta acids for control of *Varroa* mites. A registration package was submitted by Agriculture and Agri-Food Canada's Pest Management Centre to Pest Management and Regulatory Agency in July 2017. A regulatory decision has not yet been made but is expected within 18 months of the submission date.
3) Evaluated two methods of liquid application and four vapourisers for oxalic acid control of *Varroa* mites. This research is on-going and the results from this research will be used to update recommendations to beekeepers.
4) Investigated factors affecting *Nosema* prevalence and severity including application timing for fumagillin to treat *Nosema* under Alberta conditions with various management systems, with additional results forthcoming.
5) Initial lab and field screening of new antibiotics to use for *Nosema* treatment, and identification of artemisinin as a potential candidate of note.

These new miticides and antibiotics to manage *Nosema* and *Varroa* are vital to developing effective control strategies in the long term. Alberta beekeepers may have alternative options for treatment rotations which will reduce the rate at which *Varroa* mites develop resistance to chemical controls. Alberta beekeepers will continue to be able to produce quality honey that meets consumers' expectations and export market demands, and to provide pollination services to the crop industry. We have also provided data for the registration of the miticide HopGuard II, and explored alternative application methods for the registered mite control oxalic acid.

Our innovation in developing successful new miticides will have impacts that reach beyond Alberta's borders. Beekeepers across the world are looking for new options for mite control. If successful, the newly developed mitcides could be marketed globally and help in *Varroa* management systems.

Section E: Contribution to training of highly qualified personnel

Highly qualified personnel involved in the project included:

Alberta agriculture and Forestry
Dr. Shelley Hoover, Research Scientist and Apiculture Unit Head
Dr. Medhat Nasr, Provincial Apiculturist
Dr. Rassol Bahraini, Research Scientist
Dr. Tom Thompson, Research Scientist
Ms. Samantha Muirhead, Senior Technologist and Acting Provincial Apiculturist
Ms. Olivia Hares, Technologist
Ms. Cassandra Docherty, Technologist
Ms. Michelle Fraser, Technologist
Mr. Glyn Stephens, Inspection Team lead and Technologist
Ms. Mellissa Howard, Bee inspector
Mr. Maksat Igdyrov, Technologist
Ms. Sarah Waterhouse, Technologist
Ms. Alexandra Panasiuk Inspection Team lead
Ms. Karlee Shaw, Technologist
Ms. Sian Ramsden Technologist
Ms. Emily Olson, Bee inspector and Technologist
Mr. Eric Jalbert. Bee inspector and Technologist
Mr. Jeff Kearns, Technologist
Mrs. Parisa Fatehmanesh, Technologist
Mr. Jared Amos
Mr. Paul Schermund
Mr. Derek Rennie, Technologist

University Of Manitoba
Dr. Robert Currie, Professor
Ms. Rosanna Punko, M. Sc. Candidate

Guelph University
Dr. Ernesto Guzman-Novoa

Section F: The next steps

Objective 1 and 2: The process of screening a large number of chemicals having potential Varroacidal activity or efficacy against *Nosema* is costly and time consuming, and requires uncommon areas of expertise. These barriers have deterred private chemical producers and research companies from examining potential *Varroa* control compounds. Moreover, chemical companies are reluctant to investigate many of these existing registered compounds to expand labels for use in honey bees or develop new products specific to bees because of the potential high costs, and uncertain gain due to the small beekeeping market in comparison to other main agricultural crops. Some compounds with known activities against mites have gone without testing against *Varroa* mites and safety to honey bees. Our research project provides initial evidence to present to the manufacturers of the promising compounds to encourage them to pursue this line of research and eventual registration. AF will work through internal processes to pursue contacting the registrants of the FP / AIs for *Varroa* and *Nosema* control. Promising treatments should be further field tested in colony level trials across management systems and using various treatment timings. In addition, scientific articles will be written based on the experiments for objectives 1 and 2, making the results available to researchers around the world.

Our evaluation of oxalic acid applied in cardboard strips and Scott® blue shop towels as described by Maggi et al. (2015) and Oliver (2017 a, b, and c) under Alberta conditions showed that these methods have potential to control mites in the fall when minimal brood or no brood is found in bee colonies. Further research is required to refine application methods, to determine the potential for side effects on honey bees through the season, to examine residues in honey, and provide treatment recommendations for OA use in Alberta. With further research, registration of additional OA methods could be pursued.

Objective 3: *Nosema* samples from 2017 and spring 2018 need to analyzed for mean spore abundance and species. Also, brood photos from spring 2018 need to be processed. This will allow for the thorough and complete analysis of all treatments as well as wintering method for the 2017-18 experiment. The 2018-19 experiment is currently ongoing and will be completed in spring 2019. *Nosema* samples and brood photos from the rest of 2018 also require processing. Once all sample processing is complete, we will be able to provide management recommendations to beekeepers, and Ms. Punko's MSc. thesis on the research will be publicly available from the University of Manitoba.

Section G: Research Team Signatures and Employers' Approval

By signing as representatives of the research team leader's employing organisation and/or the research team member's(s') employing organisation(s), the undersigned hereby acknowledge submission of the information contained in this final report to the funder(s).

Team Leader's Organisation

Team Leader	
Name: Dr. Shelley Hoover	**Title/Organisation:** Apiculture Unit Head, Alberta Agriculture and Forestry
Signature: Shoover	**Date:** January 31, 2019
Team Leader's Employer's Approval	
Name: Dr. David Feindel	**Title/Organisation:** Director Plant and Bee Health Surveillance Section, Alberta Agriculture and Forestry
Signature: David Feindel	**Date:** Feb 4/19

Appendix 1 Additional Experiments Testing HopGuard™ (2016)

Previous research indicated that the active ingredient in HopGuard™, hop beta acids, was effective at killing mites, but the delivery method needed improvement (Vandervalk et al. 2014). When HopGuard™ was applied using cardboard strips, mite mortality declined after 3 days of treatment. It was found that the bees were removing the strip from the hive within three days of placement. This limited the exposure time and reduced the efficacy of HopGuard™.

In this study, the cardboard strip was replaced with corrugated cardboard, a more durable material, to increase exposure time to HopGuard™ and increase the efficacy of the treatment. The results from this showed this material was more effective at killing mites than the original substrate. A new label was developed for HopGuard II, and the results of the experiments were compiled to prepare a registration package to the Pest Management Regulation Agency (PMRA). As of January 2019, HopGuard II is still in the review process.

Appendix 2 Additional Oxalic Acid Experiments

Introduction

The control of the *Varroa* mite is a critical challenge to the Alberta beekeeping industry due to the limited availability of effective treatment options. Apivar® (Amitraz) is an effective miticide against *Varroa*, however, resistance has been reported, and alternatives are required for the continued and sustainable treatment of *Varroa*-infested honey bee colonies.

Oxalic acid (OA) has proven to be effective as a miticide against *Varroa* mites. The efficacy ranges from 90% to 98% in controlling *Varroa* mites when applied to a broodless honey bee colony (Charrière 2001, Radetzki 2001, Nanetti et al. 2003). Oxalic acid dihydrate is currently registered in Canada as a *Varroa* mite control product (Registration number 29575, 29576, Pest Control Products Act). There are three main methods of application: the drip/trickle method, the spray method, and the sublimation/evaporation method. Rademacher and Harz (2006) reviewed OA applications against the *Varroa* mite and showed that geographic area, climate, adult bee and brood population, *Varroa* mite infestation level, application method, and beekeeping practices all affect the efficacy of OA. It is therefore essential that currently available methods of OA application are assessed, and recommendations for treatment of honey bee colonies with *Varroa* mites in Alberta are developed.

2.1. Comparison of two new application methods of oxalic acid solutions

This project was inspired by beekeepers who thought that oxalic acid (OA) application in a strip or paper towel could be easily adopted in their fight to control *Varroa* mites. Researchers in Argentina created a new method of applying oxalic acid to the colony that prolonged colony exposure to the acid (Maggi et al. 2015). This method consists of soaking strips of chipboard in an oxalic acid glycerol solution and applying 1 strip per 5 frames of bees (between frames) for a maximum of 4 strips in the colony. Strips were left in the colony for 42 days. Colonies received treatments in the Argentinian summer (January to April) when brood was present and in the autumn (May to June) at 3 different locations. The average efficacy of the strips was 94% and 92.8% in the two summer locations and 92.8% in the autumn location (Maggi et al. 2015). Maggi et al. (2015) found no increase in oxalic acid content in honey (which is found naturally) bees wax or bees, and no negative effects to colony population was detected.

In 2017, Oliver (2017 a, b, and c) modified the Argentinian method by soaking the blue Scott® shop towels in glycerol, oxalic acid, and water. However, the efficacy of these newly developed methods is unknown under conditions found in Alberta. These experiments were undertaken to investigate the efficacy of these unregistered acaricide treatments.

Methods

The study was conducted in the fall of 2017. The mite infestation levels were not significantly different among treatments, including the non-treated control at the start of the testing process Pre-treatment assessments of adult bee and brood bee population

and Varroa infestation were performed. To evaluate the efficacy of treatments, sticky traps were replaced every 1 - 7 days during the trial as required. Twenty-one double brood chamber honey bee colonies with an average mite infestation level of 17.8% were divided into three groups, and randomly assigned to experimental treatments.

One treatment group of colonies received oxalic acid (OA) in cardboard strips as described by Maggi et al. (2015). The second group had the Scott® blue shop towel treatment as described by Oliver (Oliver 2017a, 2017b), and the third group was an untreated control. Five g OA was added to the 10 ml glycerine at approximately 65 °C. The cardboard strips were cut and a hole was punched in top of each strip for toothpick hanging. Strips were soaked in solution in a Rubbermaid tub. To do this, a cool OA solution slightly was poured evenly over strips and strips were allowed to absorb OA solution for 24 h under fume hood at room temperature. The treated strips were hung between frames in colonies.

The Scott® blue shop towels were provided to the colonies based on Oliver's method (Oliver 2017a). Pieces of towels were soaked in the OA solution (12 g OA + 10 ml water + 13 ml glycerin) for 24 h. They were placed flat in between the two brood chambers in the honey bee colonies.

Approximately 300 bees were sampled from each colony (1) before and after treatment, (2) before colonies were sublimated with oxalic acid, (3) before Apivar was applied to colonies, and (4) when Apivar was taken out of the hives. The collected samples were stored in 70% ethanol and shaken on an orbital shaker for 15 minutes. Mite and bees were counted in order to calculate the mite infestation.

Daily mite mortality was determined using sticky traps as described above. After 36 days of treatment, a finishing treatment using Oxalic acid sublimation followed by Apivar® application was used to determine the efficacy of tested methods.

Statistical analysis

The experimental honey bee colonies (n=21) were arranged in a randomized design with 7 replicates. The rate of daily *Varroa* mite mortality was calculated using Martin (1998) equation. The effects of treatments on mite mortality was analyzed by ANOVA using a repeated measures analysis of variance using an autoregressive heterogeneous covariance structure, with treatment as main effects, colonies as subjects, and day as a repeated measure. (PROC MIXED, SAS Institute Inc. 2011).

Proportions were arcsine transformed prior to analysis to improve normality and homogeneity of variance (Snedecor and Cochran 1980). All data are presented as untransformed means. Where significant interactions were observed, treatment means were compared using Bonferroni adjustment. A before and after control impact (BACI) design was used to assess the effects of treatments on Varroa mite abundance through the experiment, where treatments were treated as main effects, and period was treated as repeated measures with colony as the subject (Stewart-Oaten et al. 1986).

Results

After 36 days of treatment with the different oxalic acid application methods, there were

significant differences between the levels of mite infestation before and after the experiment. When the changes in mite level of colonies in treatment groups was calculated after using oxalic acid and Apivar® as finishing treatments, the mite level in strips and Scott® blue shop towels decreased 94.68% and 89.53%, respectively. In contrast, mite levels increased 48.61% in the control group ($F=14.55$, d.f.= 5, 14, $p<0.0001$) (Fig. A2.1.).

There was also significant difference in the daily mite drop as collected on sticky traps for tested treatments using repeated measure ANOVA ($F=13.65$, d.f.= 42, 206, $p<0.0001$) (Fig. A2.2). The daily mite drop was not significantly different between the control and Scott® blue shop treatment. (Fig. A2.2). Cumulative daily mite drop was significantly higher for cardboard strips than for the control or Scott® blue shop towel method ($F=13.93$, d.f.= 2, 16, $p= 0.0003$) (Fig. A2.3).

Discussion

Based on sticky trap mite fall counts, it appeared that the efficacy of oxalic acid applied in blue shop towels was lower and more variable in comparison to oxalic acid in strips. As the experiment continued it was discovered that mites dying in the top brood chamber fell and accumulated on the blue towels that were placed in between the two brood boxes instead of the sticky traps (Fig. A2.5). Consequently, mites on the sticky traps in these colonies did not accurately represent all dead mites during the treatment period. This may explain why there was no significant difference in daily mite drop between the control and towel treatments. Despite this low mite drop, there was a significant difference in mite infestation in the towel vs control colonies after treatment, adding weight to our theory that the mites were in fact dying, they were just not accumulating on the sticky boards as reliably as in the strip and control treatments. In addition, the high variability in the efficacy for Scott® blue shop towel method could be due to variable exposure of bees to the towels placed in between the two brood chambers in comparison to the strips placed in the cluster. These variations in efficacy of applying towels supports results reported by Oliver (Oliver 2017a, 2017b, 2017c).

2.2. Comparison of Oxalic Acid Sublimators

A pilot study to compare the efficacy of oxalic acid sublimation to control *Varroa* mite in Alberta

While broader studies on mite control using OA are required, at this time we have completed a pilot study on the vaporization method to guide future research, with the intent to use the results of this study and a literature review to design a larger study of OA mite control methods in Alberta.

Objectives

The objective of this pilot study was to compare different models of OA vaporizers on *Varroa*-infested colonies in late fall (November–December 2018) in Alberta. In this study, changes in *Varroa* mite infestation level, OA efficiency, negative effects of treatments on

honey bees, cost, and the time spent per treatment were assessed.

Methods
This project was designed to address effectiveness of OA vaporizers against mites and effects on bee health in Alberta.

All colonies (n= 28) in the experiment had average 8.67% mite infestation level with minimal brood. The queen status and bee population were evaluated in all colonies pre- and post-trial. The bee population area in all experimental colonies was visually inspected to estimate the percentage of bees covering each side of each frame. All colonies were then randomly assigned into three treatments with 7 replicates for each treatment. Sticky boards were placed under each experimental colony 3 days before treatment to determine the initial mite drop. Colonies were then treated on November 2, 9, 15 and 22, 2018 according to the manufacturer's protocol of each device (four applications, with a 7 day interval between treatment applications). During treatment, operators used personal protective equipment (respiratory mask, nitrile gloves, ear plugs, and safety shoes). To monitor daily mite mortality, a piece of sticky board (30X43 cm) was placed in the tray of the screened bottom board of each colony to collect dying mites that fall through the screen. Sticky traps were replaced at days 1, 3, 5 and 7 days post-treatment. The dead mites on these sticky traps will be counted, and daily mite mortality will be calculated in each test colony. Temperature of the OA aerosol cloud and muzzle of devices were measured at the time of application using visual infrared thermometer (Flir TG165). The number and size of OA particles were measured using particle counter (Handled particle counter, KANOMAX 3889, USA).

Apivar® was applied on November 29, 2018 as finishing treatment to kill the remaining mites in the colonies, and determine the efficacy of the OA treatment. A sample of 250-300 worker bees were taken from each colony before and after the experiment, and before each application of oxalic acid to evaluate the phoretic mite population. The bee samples, which were stored in 70% ethanol, will be shaken in an orbital shaker (300 rpm) for 15 minutes to determine the mite level in samples. All colonies were wrapped outdoors and the effects of treatment on winter survival will be evaluated in next spring.

Records
In this pilot trial three vaporizers were tested:

1-ProVap 110 (sideliner/Commercial vaporizer, OxoVap LLC, SC, USA)
Vaporizer took 2-5 min to reach the setting temperature (230 °C / 446 °F) depending on the ambient temperature. One gram of OA crystal (Oxalic acid dihydrate 99.6%, Medivet, AB, Canada) was loaded in a Teflon™ lid using a plastic spoon (2.5 ml) and then device was inserted in the hive entrance. Colonies were treated for 20 seconds (Fig. A2.5). The temperature of device body and OA cloud was 62.5 °C and 16.1 °C, respectively (Fig. A2.8). One colony was recorded as dead at the end of treatment period (November 29). The OA particle number (and size) measured for this device were: 27185 (0.3 µm), 26916 (0.5 µm), 24841 (1.0 µm), 2132 (3.0 µm), 67 (5.0 µm) and 1 (10.0 µm).

2-*Varroa* blaster (supplied by Terry Greidanus)

Varroa blaster took 5-15 min to reach to the setting temperature (218 °C / 425 °F) depending on the ambient temperature. Approximately 10-12 grams of OA crystal was loaded in vaporizer using a baster and the treatment colonies were exposed to the OA cloud for 10 seconds (Fig. A2.6).

3-*Varroa* Cannon (Varroa cannon, USA)
This vaporizer took 5-15 min to reach to the setting temperature (343 °C / 650 °F) depending on the ambient temperature. Approximately 10-12 grams of OA crystal was loaded in vaporizer using a baster and then OA cloud went through the treatment colonies for 10 seconds (Fig. A2.7). The temperature of device body and OA cloud was 192°C and 10.5 °C, respectively (Fig. A2.8). One colony was recorded as dead at the end of treatment period (November 29).

Analyses of mite drop count, mean abundance of mite, analysis of daily mite mortality, OA efficacy and changes in the mite infestation level are in process.

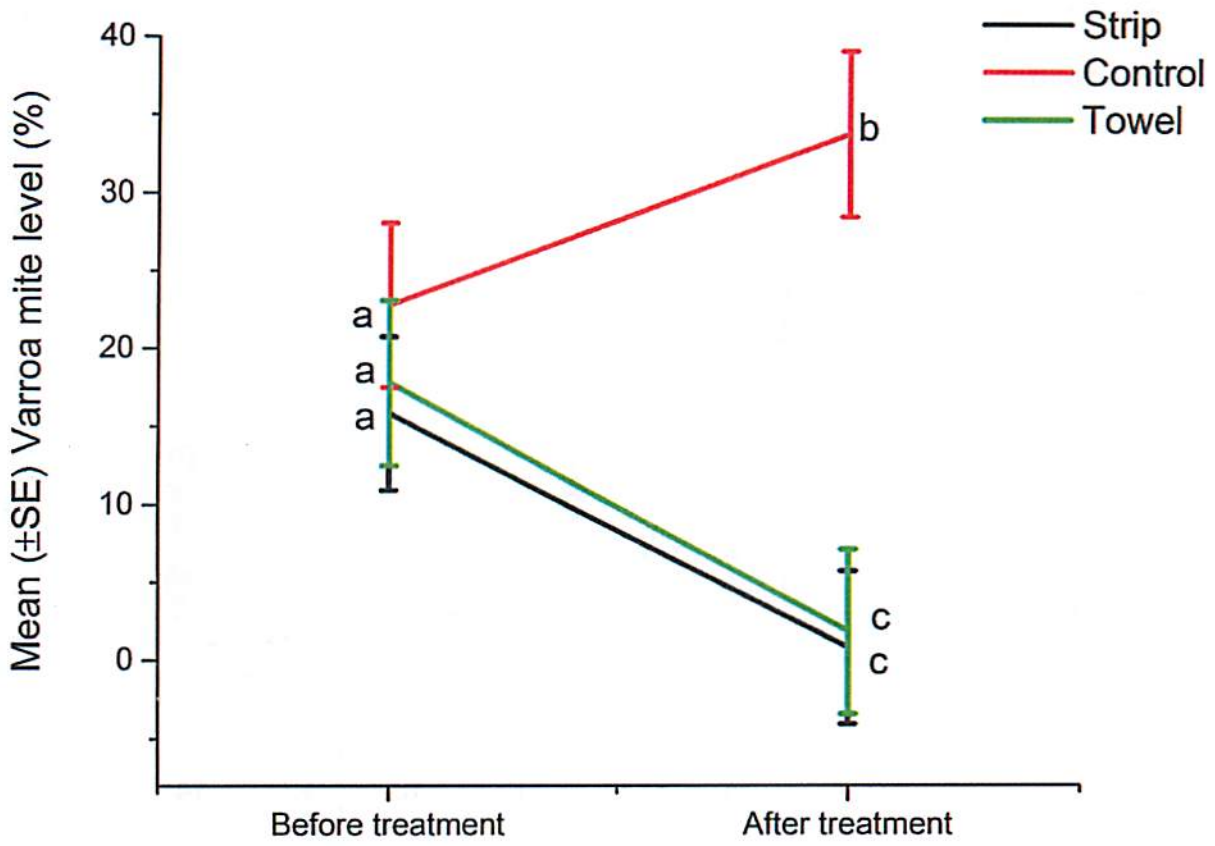

Figure A2.1. Mean (±SE) Varroa mite infestation level (%) in bee population of treated colonies before and after the experiment. The mite level in the strip and Scott® blue shop towel groups decreased 94.68% and 89.53%, respectively, however, this variable increased 48.61% in control group. Letters indicate significant differences among treatments ($P<0.05$).

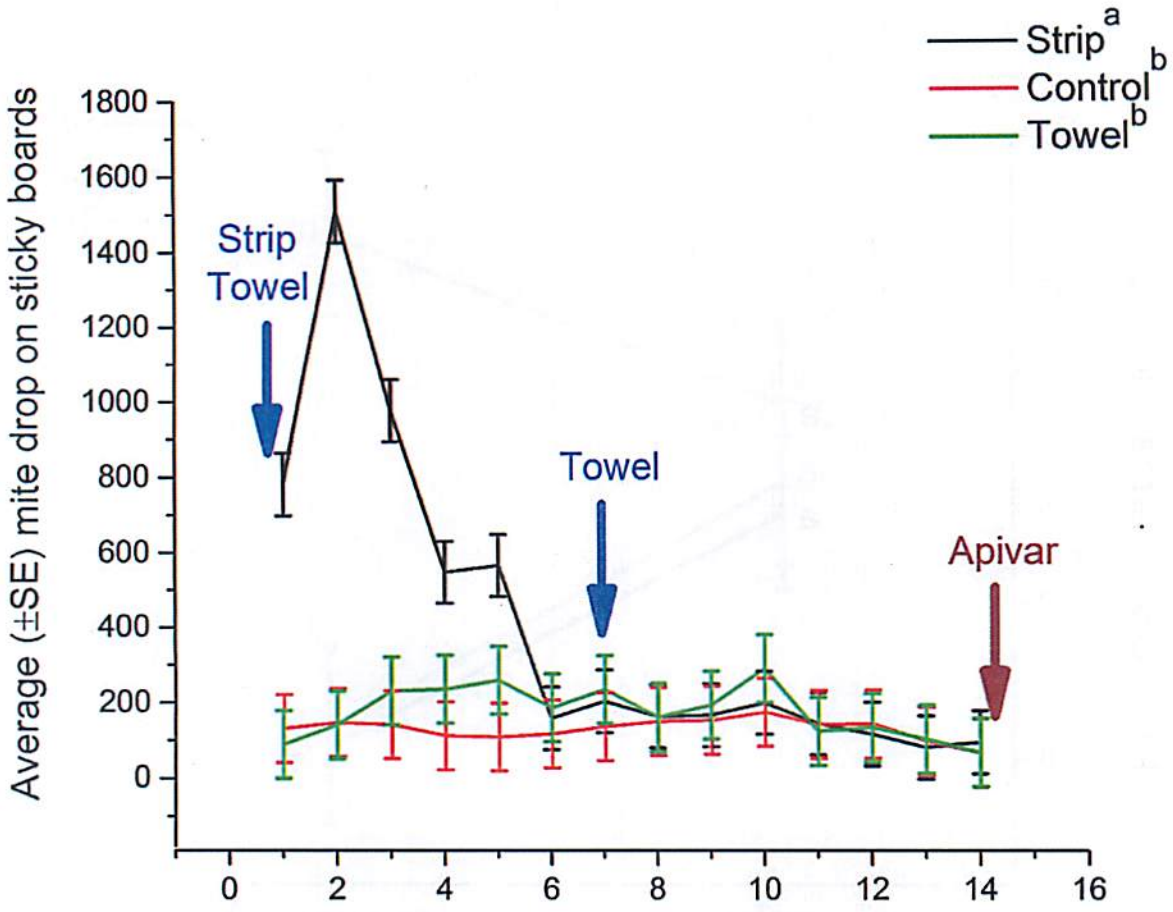

Figure A2.2. Average daily *Varroa* mite drop on sticky boards in honey bee colonies for different acaricide treatments. There were significant differences among treatments ($p < 0.0001$). The X-axis indicates the time point of sampling (Oct 10 – Nov 14, 2017). Arrows indicate treatment point for strip, towel and Apivar. Letters equal significant differences among treatments ($P<0.05$).

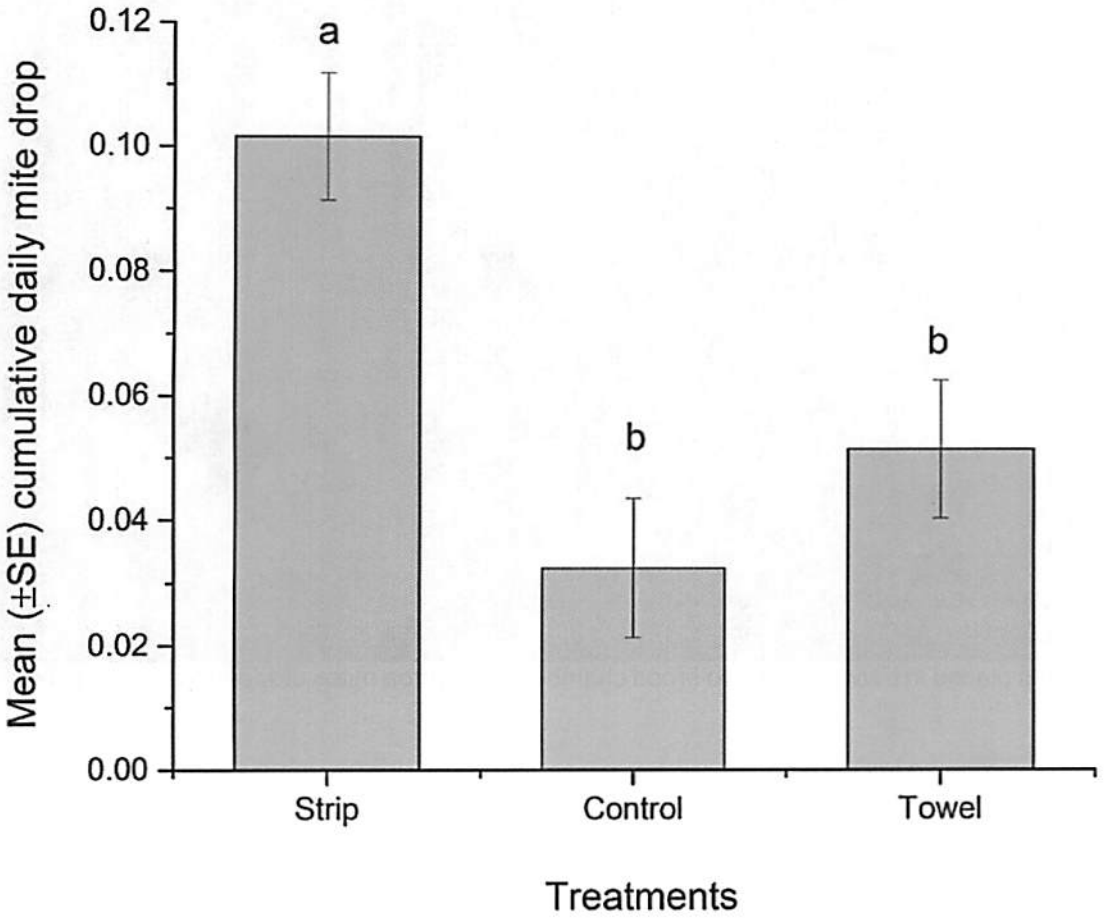

Figure A2.3. Mean (±SE) cumulative daily mite drop across three treatments of OA application. Letters equal significant differences among treatments ($P<0.05$).

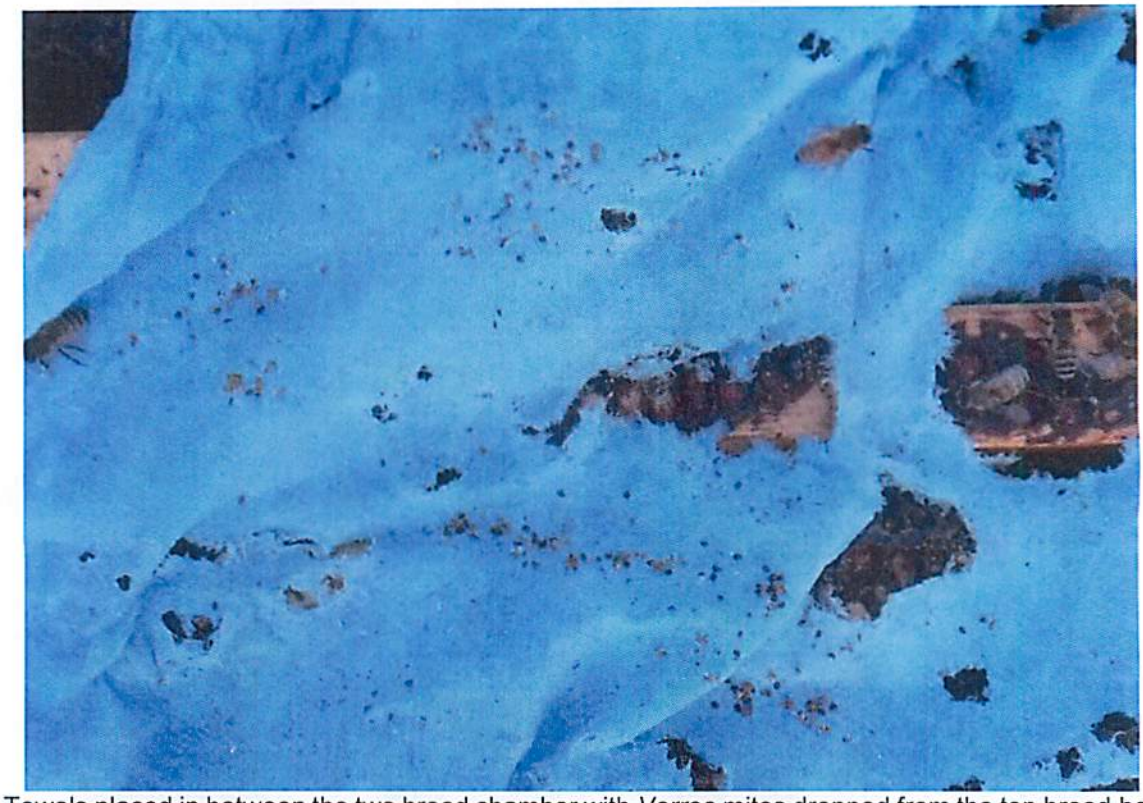

Figure A2.4 Towels placed in between the two brood chamber with *Varroa* mites dropped from the top brood box.

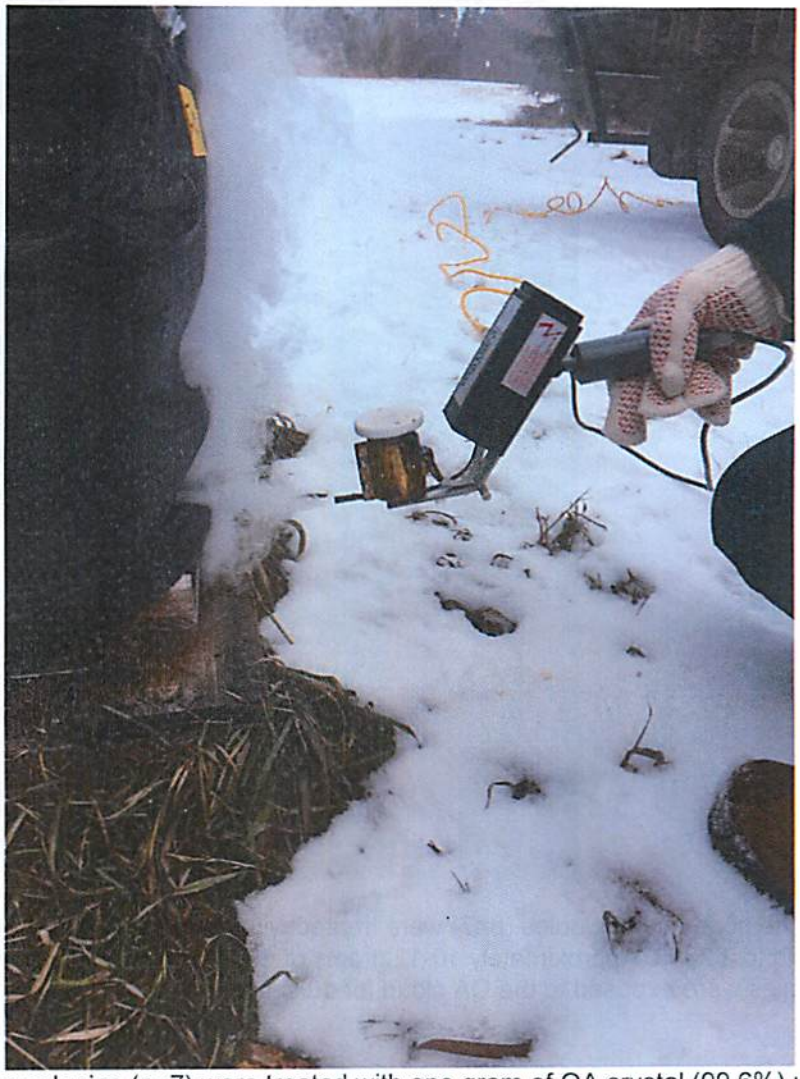

Figure A2.5. The honey bee colonies (n=7) were treated with one gram of OA crystal (99.6%) using ProVap 110 Oxalic acid vaporizer in field test 2018. OA was loaded in Teflon lid using a plastic spoon (2.5 ml) and then device was inserted in the hives' entrances. Colonies were treated for 20 seconds.

Figure A2.6. The honey bee colonies (n=7) were treated with OA crystal (99.6%) using Varroa Blaster Oxalic acid vaporizer in field test 2018. Approximately 10-12 grams of OA crystal was loaded in vaporizer using a baster and the treatment colonies were exposed to the OA cloud for 10 seconds.

Figure A2.7. The honey bee colonies (n=7) were treated with OA crystal (99.6%) using Varroa Cannon oxalic acid vaporizer in field test 2018. Approximately 10-12 grams of OA crystal was loaded in vaporizer using a baster and the treatment colonies were exposed to the OA cloud for 10 seconds.

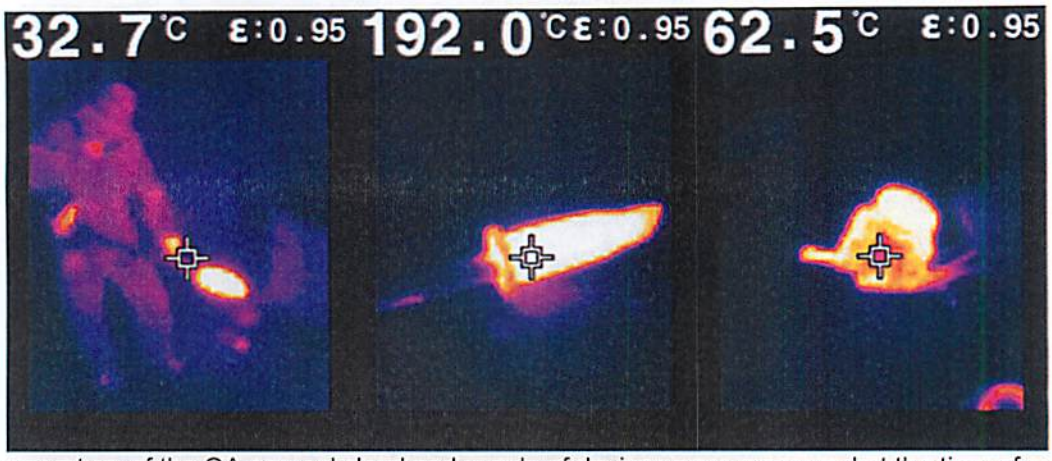

Figure A2.8. Temperature of the OA aerosol cloud and nuzzle of devices were measured at the time of application using visual infrared thermometer (Flir TG165). Photos indicate the temperature of body of vaporizers Varroa Cannon (right anc middle) and ProVap (left).

Appendix 3 Extension Activities

Extension activities are summarized in the following tables.

Year	Event Type	Number of Events per year	Total Number of Participants	Number of Participants by Industry Type
2014	Productivity Workshop			Apiculture
		9	425	Grain and Oilseed
				Pesticides
	One-on-One	425		Apiculture
				Oilseed and Grain Farming
			5	Pesticides
	Oral presentations at various meeting in Alberta, across Canada and USA	15	1900	Apiculture
			50	Oilseed and Grain Farming
			70	Pesticides
			1	Horticulture crops
			3	Public
	Newspaper article and media interviews	4		Western producers, Western grain, Edmonton Journal, the Sun
	Annual recommendation Info sheet	1	750	Apiculture
	Monthly article Newsletter	12	300	Apiculture
	Fact sheets	1		Apiculture

Year	Event Type	Number of Events per year	Total Number of Participants	Number of Participants by Industry Type
2015	Productivity Workshop	8	400	Apiculture
				Grain and Oilseed
				Pesticides
	One-on-One	425	425	Apiculture
				Oilseed and Grain Farming
		10	10	Pesticides
	Oral presentations at various meeting in Alberta, across Canada and USA	14	1600	Apiculture
				Oilseed and Grain Farming
		1	45	Pesticides
				Horticulture crops
		2	80	Public
	Newspaper article and media interviews	2		Western producers, Western grain, Edmonton Journal, the Sun
	Annual Reccomendation Info sheet	1	1100	Apiculture
	Monthly article Newsletter	12	1100	Apiculture
	Fact sheets			Apiculture

Year	Event Type	Number of Events per year	Total Number of Participants	Number of Participants by Industry Type
2016	Productivity Workshop	5	325	Apiculture
		1	70	Grain and Oilseed
				Pesticides
	One-on-One	320	320	Apiculture
		5	5	Oilseed and Grain Farming
			15	Pesticides
	oral presentations at various meeting in Alberta, across Canada and USA	17	1650	Apiculture
				Oilseed and Grain Farming
		2	45	Pesticides
				Horticulture crops
		3	90	Public
	Newspaper article and media interviews	3		Western producers, Western grain, Edmonton Journal, the Sun
	Annual reccomendation Info sheet	1	1250	Apiculture
	Monthly article Newsletter	12	1250	Apiculture
	Fact sheets	1		Apiculture

Year	Event Type	Number of Events per year	Total Number of Participants	Number of Participants by Industry Type
2017	Productivity Workshop	1	100	Apiculture
				Grain and Oilseed
				Pesticides
	One-on-One	300	300	Apiculture
			15	Oilseed and Grain Farming
		25	25	Pesticides
	oral presentations at various meeting in Alberta, across Canada and USA	12	1400	Apiculture
				Oilseed and Grain Farming
		2	45	Pesticides
				Horticulture crops
		5	150	Public
	Newspaper article and media interviews	4		Western producers, Western grain, Edmonton Journal, the Sun
	Annual reccomendation Info sheet	1	1450	Apiculture
	Monthly article Newsletter	7	1450	Apiculture
	Fact sheets			Apiculture

Year	Event Type	Number of Events per year	Total Number of Participants	Number of Participants by Industry Type
2018 - Estimates	Productivity Workshop	5	200	Apiculture (Pests/diseases/IPM)
				Grain and Oilseed
				Pesticides
	One-on-One	3	7	Apiculture (Pests/diseases/IPM)
				Oilseed and Grain Farming
				Pesticides
	oral presentations at various meeting in Alberta, across Canada and USA	7	300	Apiculture (Pests/diseases/IPM)
				Oilseed and Grain Farming
				Pesticides
				Horticulture crops
				Public
	Newspaper article and media interviews	1		Western producers, Western grain, Edmonton Journal, the Sun
	Annual recommendation Info sheet			Apiculture
	Monthly article Newsletter			Apiculture
	Fact sheets/Reports	4		Apiculture (Pests/diseases/IPM)

Appendix 4 Objective 1 (a-c): Developing new miticides.

Table A4.1. List of tested chemical classes and their mode of action.

Class	Mode of action	No. AIs tested	No. FPs tested	Year
Avermectins	GABA agonist	2	1	2017/2018
Benzoylacetonitriles	Inhibitor of mitochondria complex II	1	1	2017/2018
Buprofezins	Inhibitor of chitin biosynthesis	1	0	2018
Carbazates	Inhibits the respiration of mitochondria	1	1	2016/2018
Formamidines (Amitraz)	Octopamine receptor agonist	1	2	2016/2017/2018
Milbemycins	GABA agonist	1	0	2018
Organochlorines	Hyperstimulation of nerve transmission	1	0	2018
Organosulfides	Inhibitor of magnesium-stimulated ATPase	1	0	2018
Oxazolines	Growth inhibitor	1	0	2017/2018
Pyrazoles	Inhibitor of ATP synthase	3	1	2017/2018
Pyrethroids	Effect on voltage-sensitive ion channels	2	1	2017/2018
Pyridazinones	Hyperstimulation of nerve transmission	1	0	2018
Pyrroles	Effect on oxidative phosphorylation	1	1	2017/2018
Quinazolines	Inhibitor of lipid biosynthesis	1	1	2018
Quinolines	Inhibits the respiration of mitochondria	1	2	2016/2017/2018
Tetrazines	Growth inhibitor	1	1	2016/2017/2018
Tetronic acids	Inhibitor of acetyl-CoA carboxylase	3	3	2016/2017/2018
Thiazolidinones	Growth inhibitor	1	0	2018

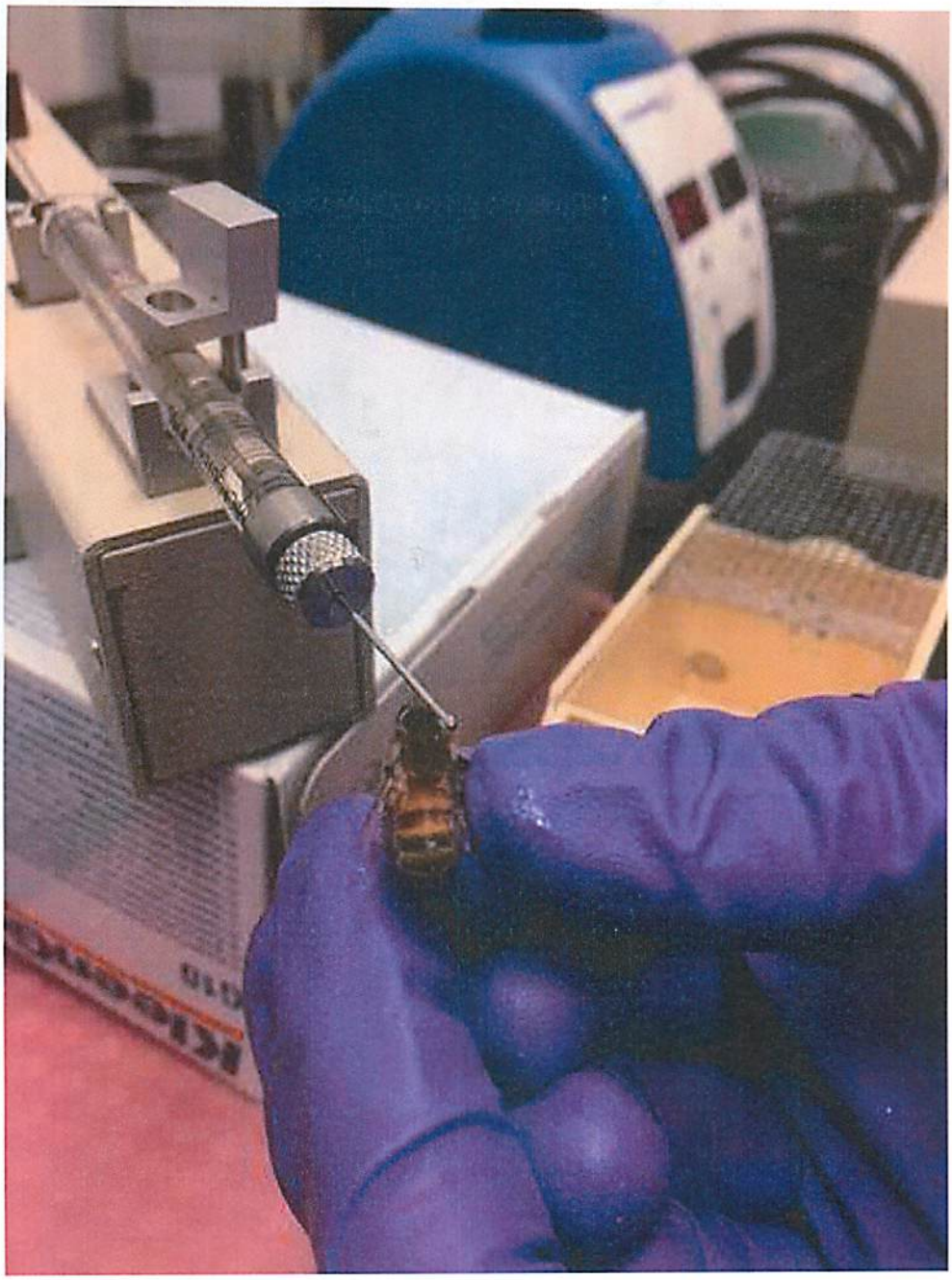

Figure A4.1. A newly-emerged worker bee is topically treated 1 µl of tested compound using Hamilton micro syringe in a precision micro-applicator. Once treated, the bees were placed in plastic cages and fed one sugar cube. The bees were incubated at 33°C for 24 hours. Bee mortality was assessed 24 h post-treatment. In this test, LD50 and bee mortality were assessed.

Figure A4.2. Treated scintillation glass vials are rotated on a cold hot dog roller under the fume hood at room temperature for 2-3 h until the solvents are completely evaporated and compounds are homogenously spread on the inner surface of vials.

Figure A4.3. To measure the mite mortality and LC50 of candidate compounds, a group of 8-10 mites are introduced into the treated scintillation glass vials (20 ml) and fed purple-eyed honey bee pupae. Prepared vials were incubated at 25°C for 6 hours (2016) or at 33°C for 4 hours (2017-2018). Mite mortality was counted 6 (2016) or 4 (2017-2018) hours post-treatment.

Figure A4.4. To measure the honey bee mortality and LC50 of candidate compounds, a group of newly-emerged adult worker bees are exposed to tested compounds in The Mason Jar Assay (2016). All prepared Mason jars with bees were incubated at 25 °C in the dark. Dead bees were counted in each jar to determine bee mortality after 24 h exposure.

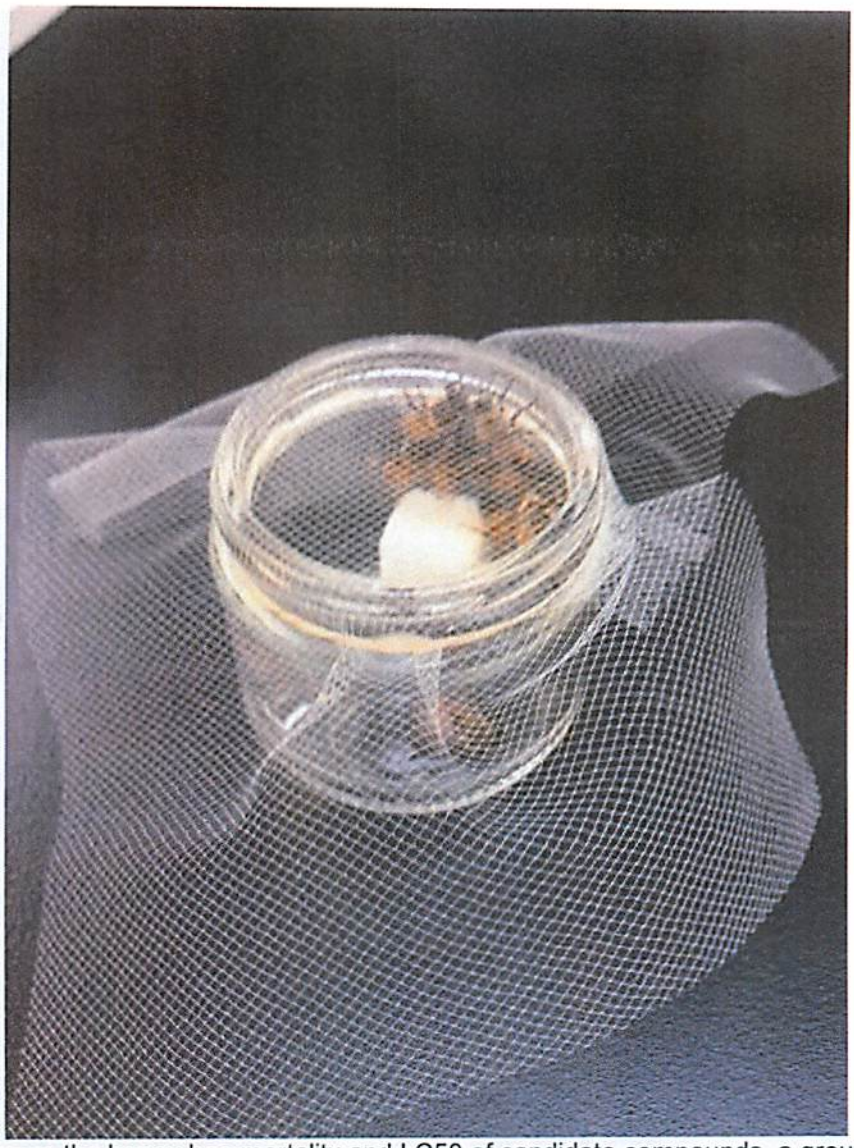

Figure A4.5. To measure the honey bee mortality and LC50 of candidate compounds, a group of 10 newly-emerged worker bees are introduced into the treated 60 ml Mason jar, and fed a sugar cube. All prepared Mason jars with bees were incubated at 25 °C in the dark. Dead bees were counted in each jar to determine bee mortality after 24 h exposure.

Figure A4.6. To evaluate efficacy of candidate compounds, in field trials, single brood chamber Langstroth colonies were treated with different doses (Table A4.3) of Tetrazine, Quinoline, and Tetronic acids I and III. A Gary dead-bee trap was installed on each colony to collect dead bees (2016).

Figure A4.7. To evaluate efficacy of candidate compounds in field trials, single brood chamber Langstroth colonies were modified to three compartments. Compartments were treated with different doses of compounds. Each compartment accommodated 3 frames of bees and one new mated queen. In 2017, tetronic acid I and II, pyrazole I, pyrrole and benzoylacetonitrile; and in 2018, tetronic acid II, pyrazole I and benzoylacetonitrile were tested.

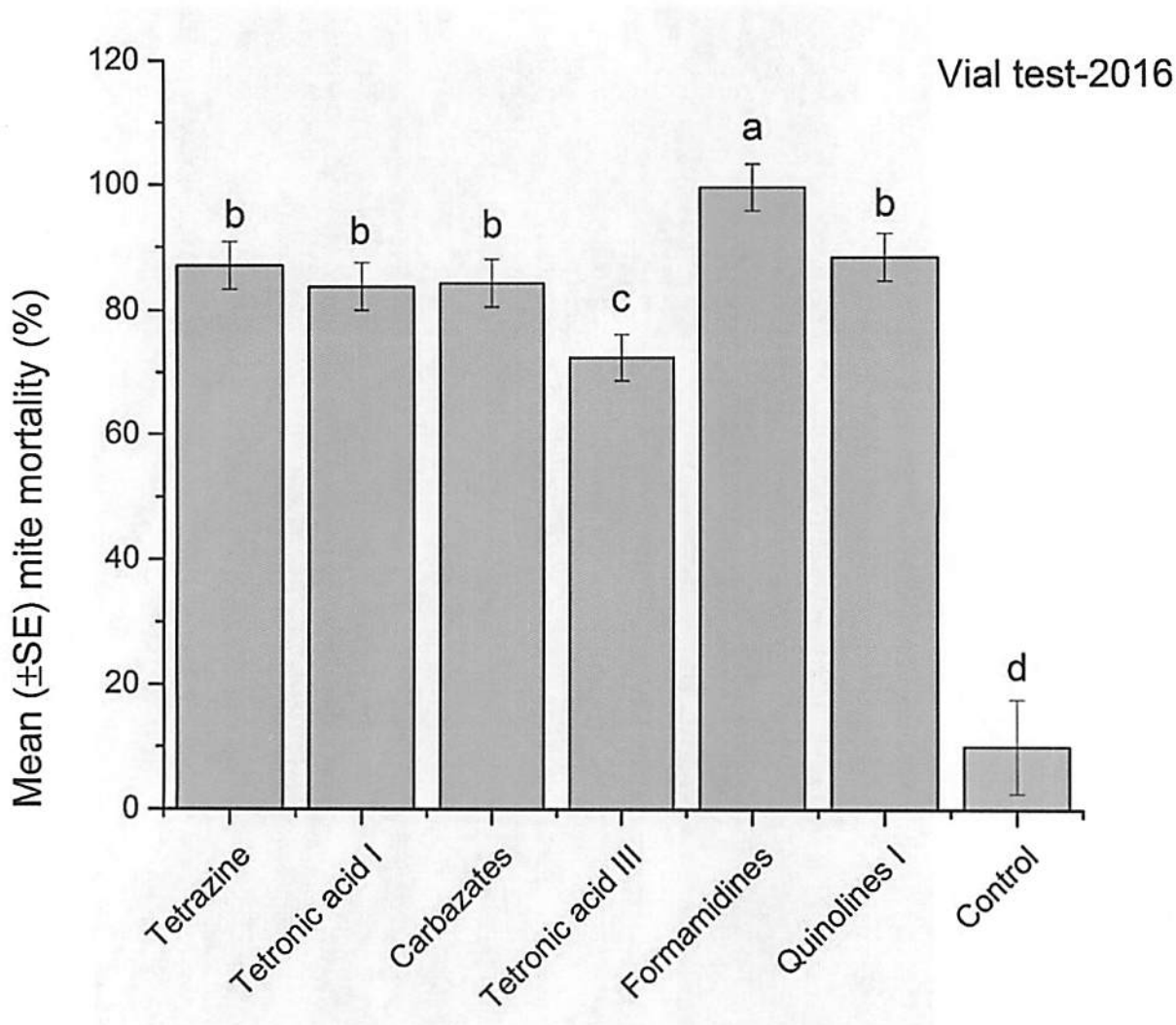

Figure A4.8. Mean (±SE) percentage mite mortality during 24 h exposed to tested formulated products in glass vials under laboratory conditions. Means with the same letter among treatments are not significantly different ($p<0.05$) (2016).

Table A4.2. Lethal concentration values (LC_{50}) for 24 h responses of mites and bees to tested formulated products (FP) and active Ingredients (AI), 2016-2108.

FP/AI	Calss	LC50 mite	95%CL	95%CL	LC50 Bee	95%CL	95%CL
AI	Avermectine I	1.45261	.	.	4.22E-06	5.32E-07	2.36E-05
FP	Avermectine I	1.76E-06	3.80E-09	3.64E-05	5.15E-13	.	.
AI	Benzoylacetonitrile	3691	1.06781	1.45E+29	3.31E4(LC90)	.	.
FP	Benzoylacetonitrile	3.06E+70	.	.	2.60E+14	.	.
AI	Carbazate	2.06E-06	3.99E-12	0.000142	.	.	.
FP	Carbazate	0.00661	0.000718	0.02671	.	.	.
FP	Formamidine	0.00887	.	.	.	.	.
AI	Formamidine	1.52E-10	8.18E-70	1.54E-07	3.86E-05	2.76E-06	0.00033
AI	Organochlorine I	0.0036	0.00076	0.01995	0.0002974	4.65E-05	0.00119
AI	Oxazoline	7.87E+27	.	.	0.00178	.	
FP	Pyrazole I	3.23E-06	2.36E-08	2.32E-05	1.40E-04	1.16E-05	0.00125
AI	Pyrazole I	0.00424	0.00008	15.70222	0.0000526	4.89E-06	0
AI	Pyrazole II	0.0001994	3.73E-06	0.4873	2.72E-07	9.18E-14	0.000036
AI	Pyrazole III	0.0011	0.000173	0.02983	0.0005797	0.000115	0.00192
AI	Pyrethroid I	0.00148	0.00037	0.00983	0.000055	6.78E-06	0.000238
AI	Pyrethroid II	5.28E-02	.	.	7.64E-08	3.08E-17	5.78E-06
FP	Pyrethroid II	9.12E-06	7.57E-06	0.000011	2.68E-07	2.13E-09	4.08E-06
AI	Pyrrole	3.03E-06	1.23E-12	2.76E-05	2.78E+05	4.84E-07	0.000288
FP	Pyrrole	1.93E-07	2.81E-25	1.61E-05	1.44E-05	1.14E-06	8.07E-05
AI	Quinazoline	0.0007495	9.55E-05	0.07072	0.00193	0.00016	0.01481
FP	Quinoline I	0.00273	4.46E-05	0.01897	.	.	.
AI	Quinoline I	9.52E+23	.	.	1.58E-11	2.09E-125	2.50E-07
FP	Quinoline II	1.12E-19	.	.	1.46E-12	.	.
FP	Tetrazine	0.00162	1.51E-05	0.0162	.	.	.
AI	Tetrazine	4.55E+23	.	.	9.69E-20	.	.
AI	Tetronic acid I	5.81E-61	.	.	2.27E-45	.	.
FP	Tetronic acid I	0.0043	0.000273	0.01746	.	.	.
FP	Tetronic acid II	7.17E-25	.	.	4.02E-03	.	.
AI	Tetronic acid II	1.78E+22	.	.	4.21E+34	.	.
FP	Tetronic caid III	0.00944	.	.	.	.	.
AI	Tetronic caid III	7.03E-06	3.60E-08	0.000143	.	.	.
AI	Thiazolidinone	1399377	.	.	3.42E-06	.	

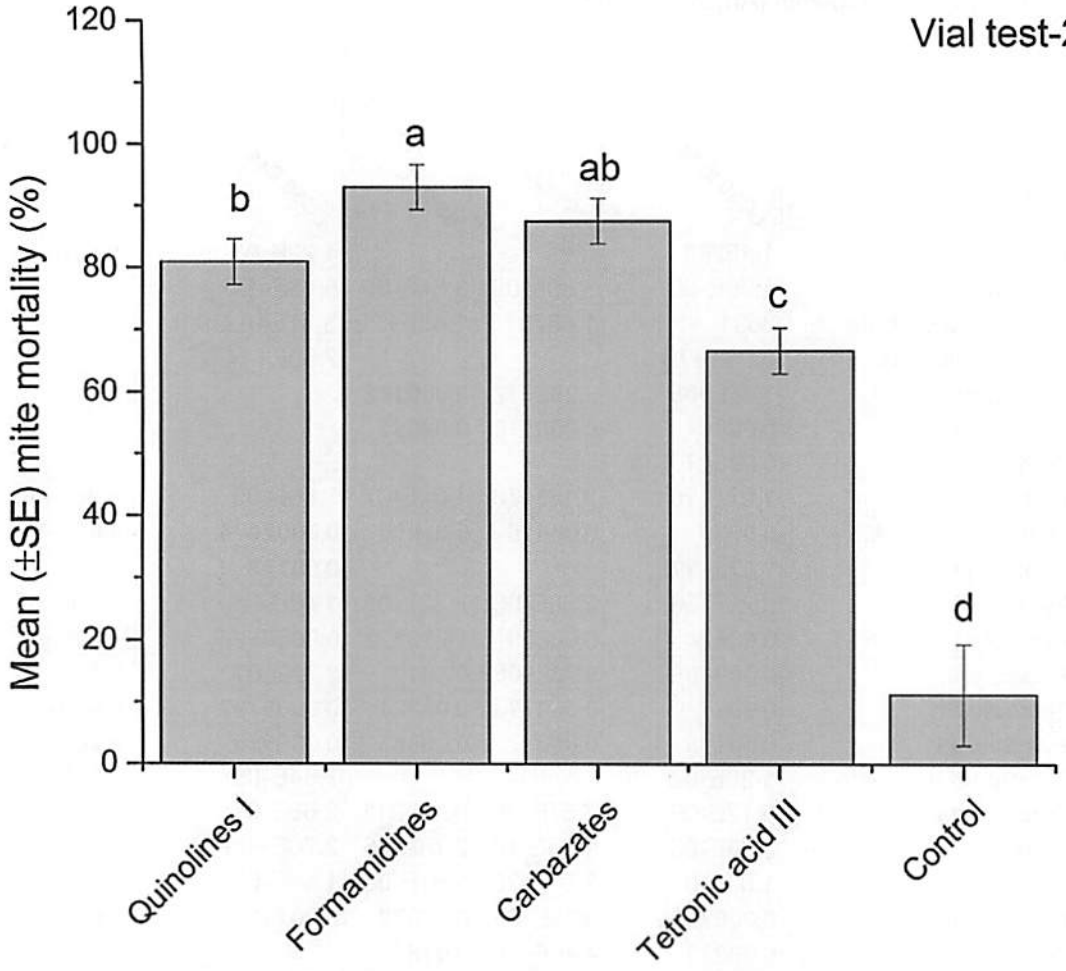

Figure A4.9 Mean (±SE) percentage mite mortality during 24 h exposed to tested active ingredients in glass vials under laboratory conditions. Means with the same letter among treatments are not significantly different ($p<0.05$) (2016).

Table A4.3. Evaluation of the efficacy of formulated products and their active ingredients against *Varroa* mites and side effects on bees under field condition in 2016.

Treatment	Average daily mite mortality (±SE)	Average daily bee mortality (±SE)	Concentration or dose	AI mg/colony
Formulated Products				
Tetrazine	69.69±31.77	104.67±155	1%	0.324
			5%	1.62
			10%	3.24
			15%	4.86
			20%	6.48
Tetronic acid I	59.01±31.77	143.00±15.55	1%	0.324
			5%	1.62
			10%	3.24
			15%	4.86
			20%	6.48
Quinoline	54.25±31.77	180.50±19.04	1%	0.324
			5%	1.62
			10%	3.24
			15%	4.86
Formamidine	232.21±55.03	110.00±26.92	3%	0.972
Active Ingredients				
Quinoline	67.96±67.77	64.67±17.80	50 mg	50
			100 mg	100
			150 mg	150
Carbazate	59.13±67.77	68.33±17.80	50 mg	50
			100 mg	100
			150 mg	150
Tetrazine	30.47±83.00	29.50±17.80	50 mg	50
			100 mg	100
			150 mg	150
Tetronic acid I	116.67±67.77	30.33±17.80	50 mg	50
			100 mg	100
			150 mg	150
Tetronic acid III	50.02±67.77	64.33±17.80	50 mg	50
			100 mg	100
			150 mg	150
Apivar®	253.96±39.13	44.56±10.28	3.33%	1000
Control	69.45±47.92	26.83±12.60	-	-

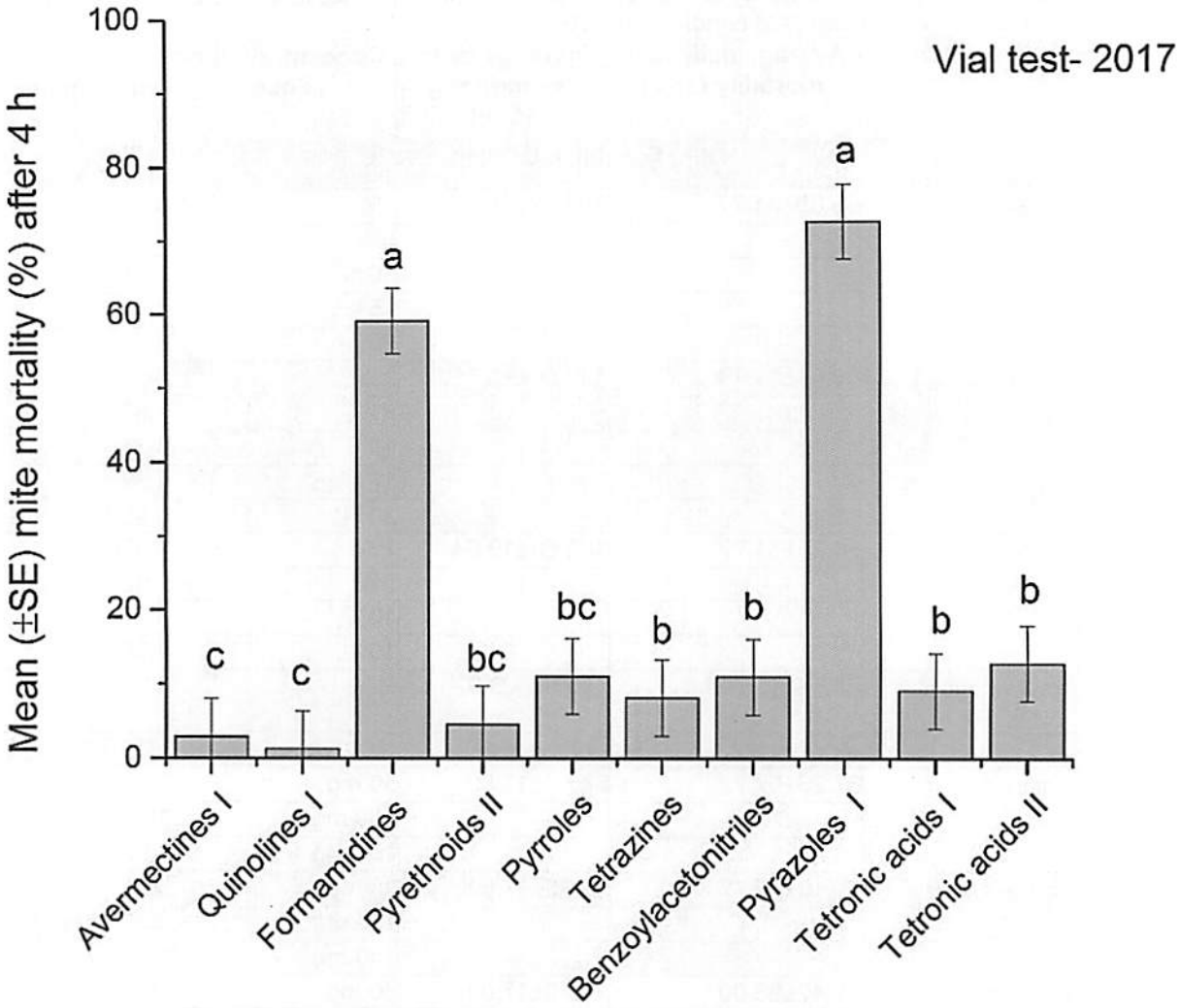

Figure A4.10. Mean (±SE) mite mortality exposed to tested active ingredients after 4 h. Means with the same letter among treatments are not significantly different ($p<0.05$) (2017).

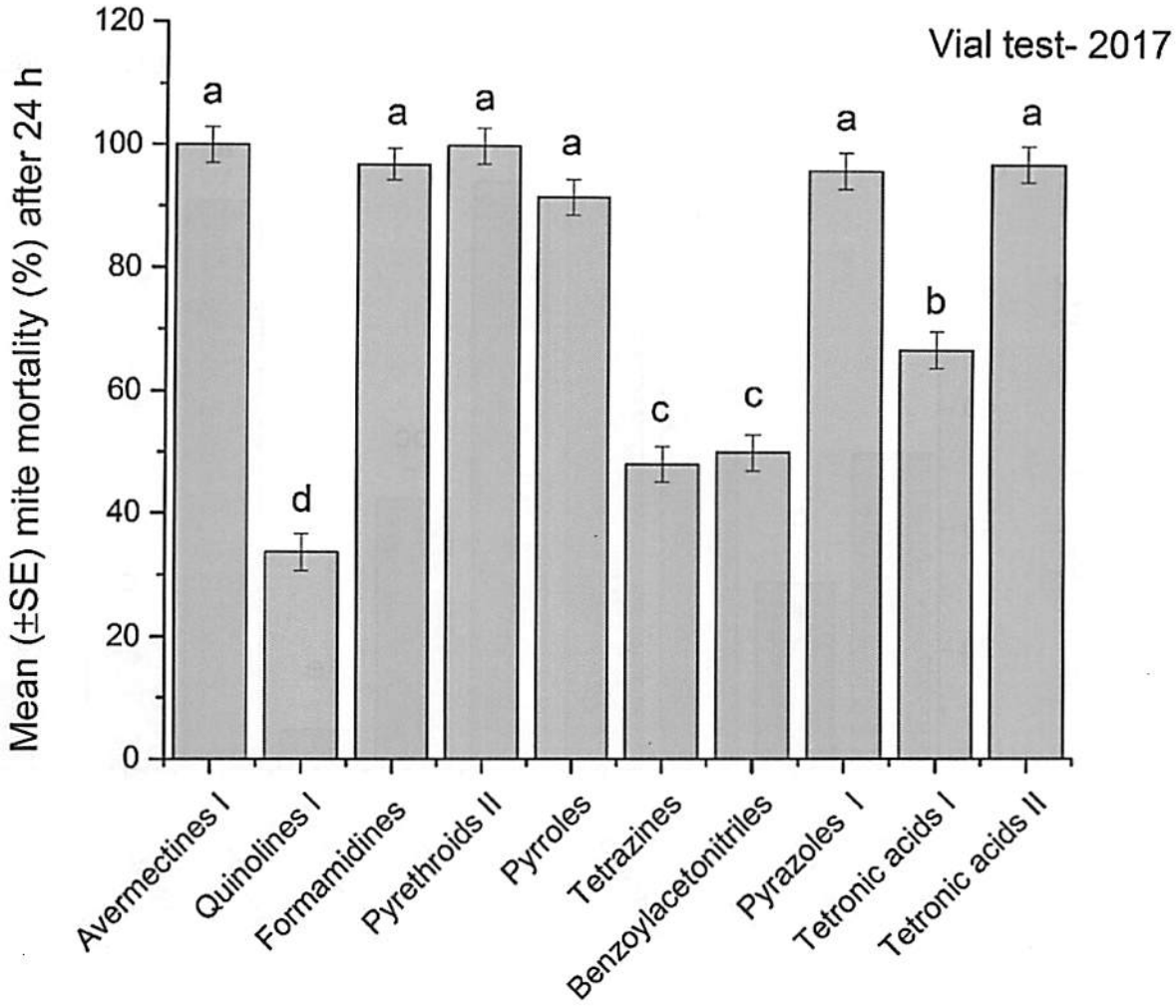

Figure A4.11. Mean (±SE) mite mortality exposed to tested active ingredients after 24 h. Means with the same letter among treatments are not significantly different ($p<0.05$) (2017).

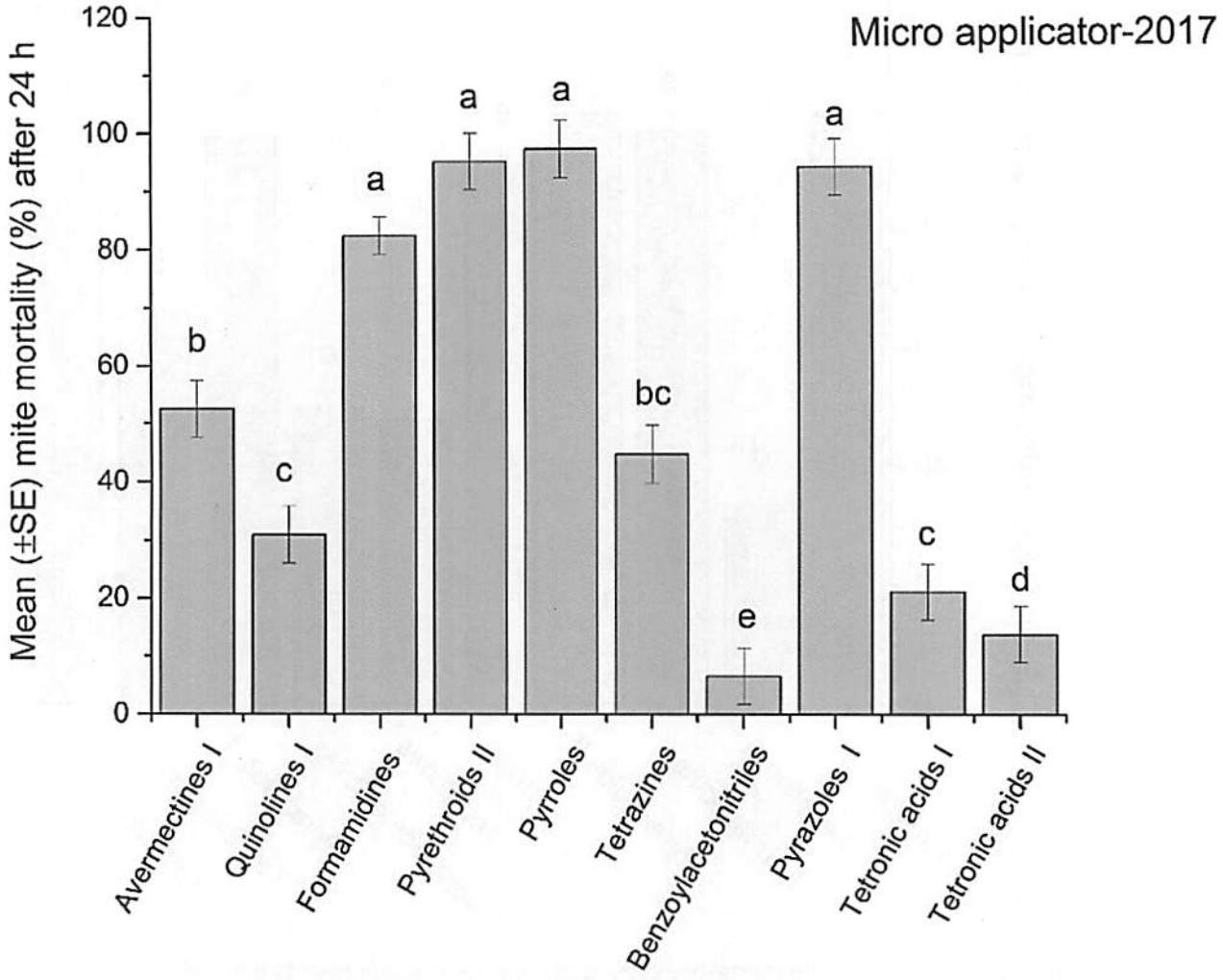

Figure A4.12. Mean (±SE) mite mortality exposed tested active ingredients using the micro applicator after 24 h. Means with the same letter among treatments are not significantly different ($p<0.05$) (2017).

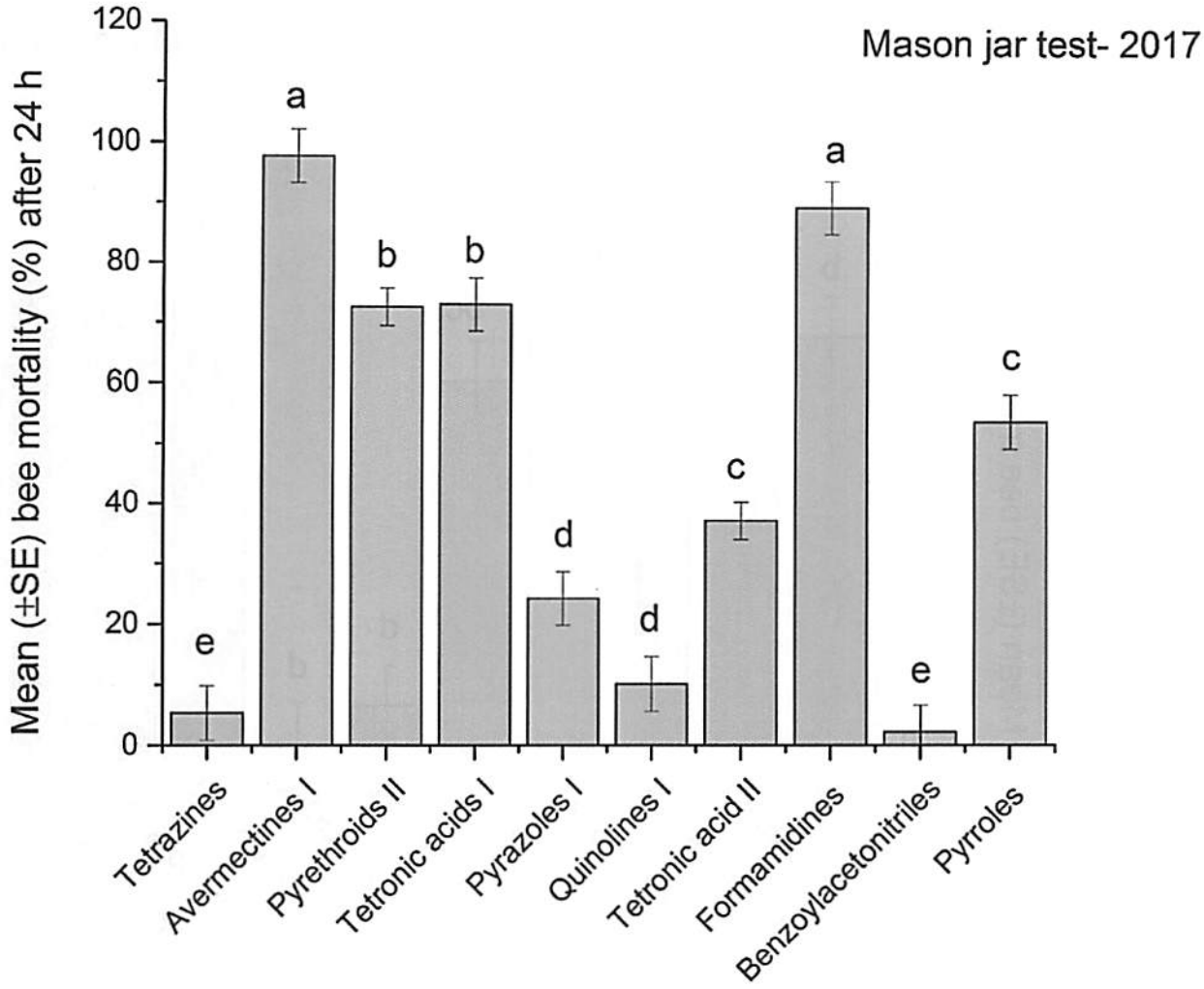

Figure A4.13. Mean (±SE) bee mortality exposed to tested formulated products after 24 h. Means with the same letter among treatments are not significantly different (p<0.05) (2017).

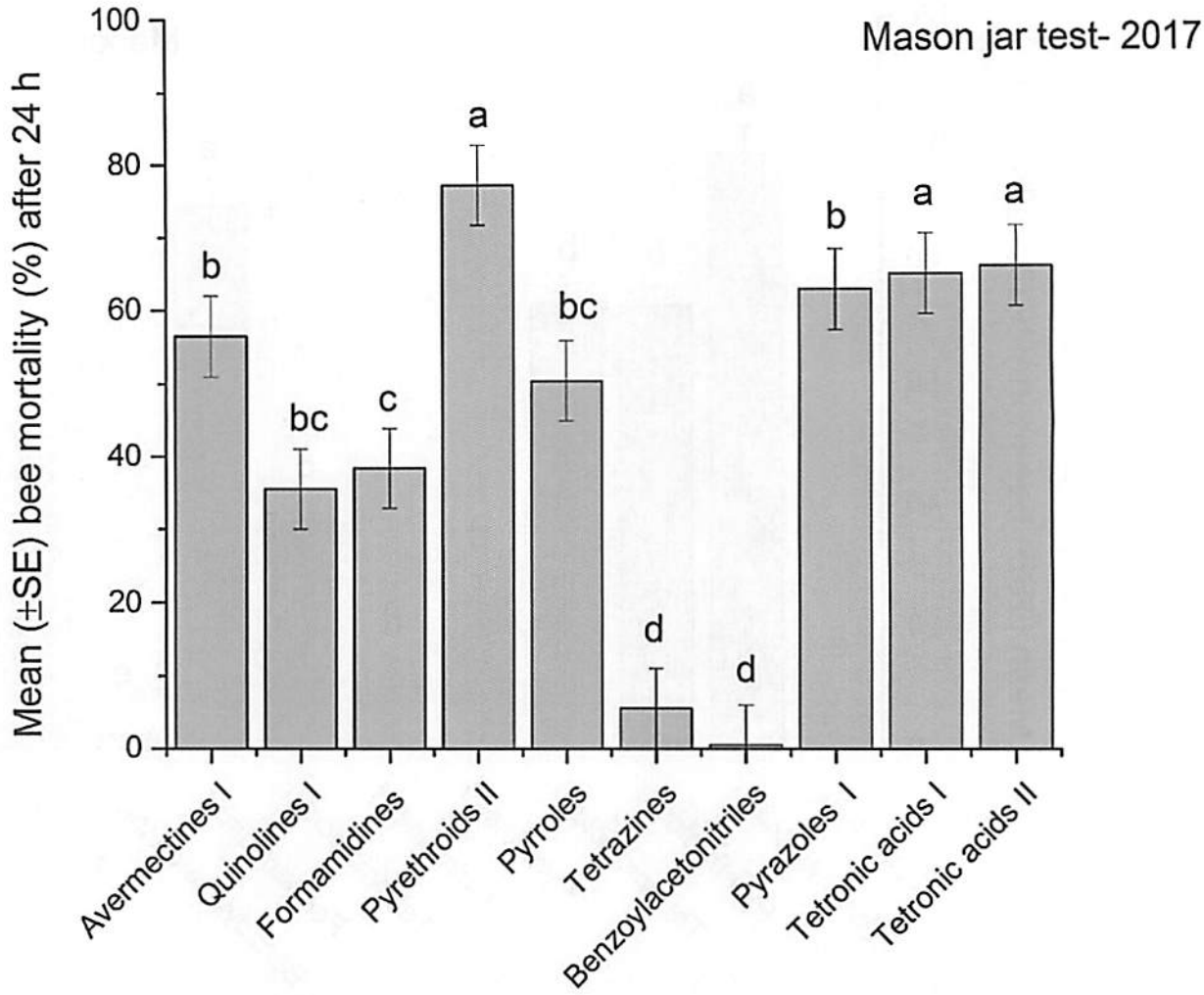

Figure A4.14. Mean (±SE) bee mortality exposed to tested active ingredients after 24 h. Means with the same letter among treatments are not significantly different ($p<0.05$) (2017).

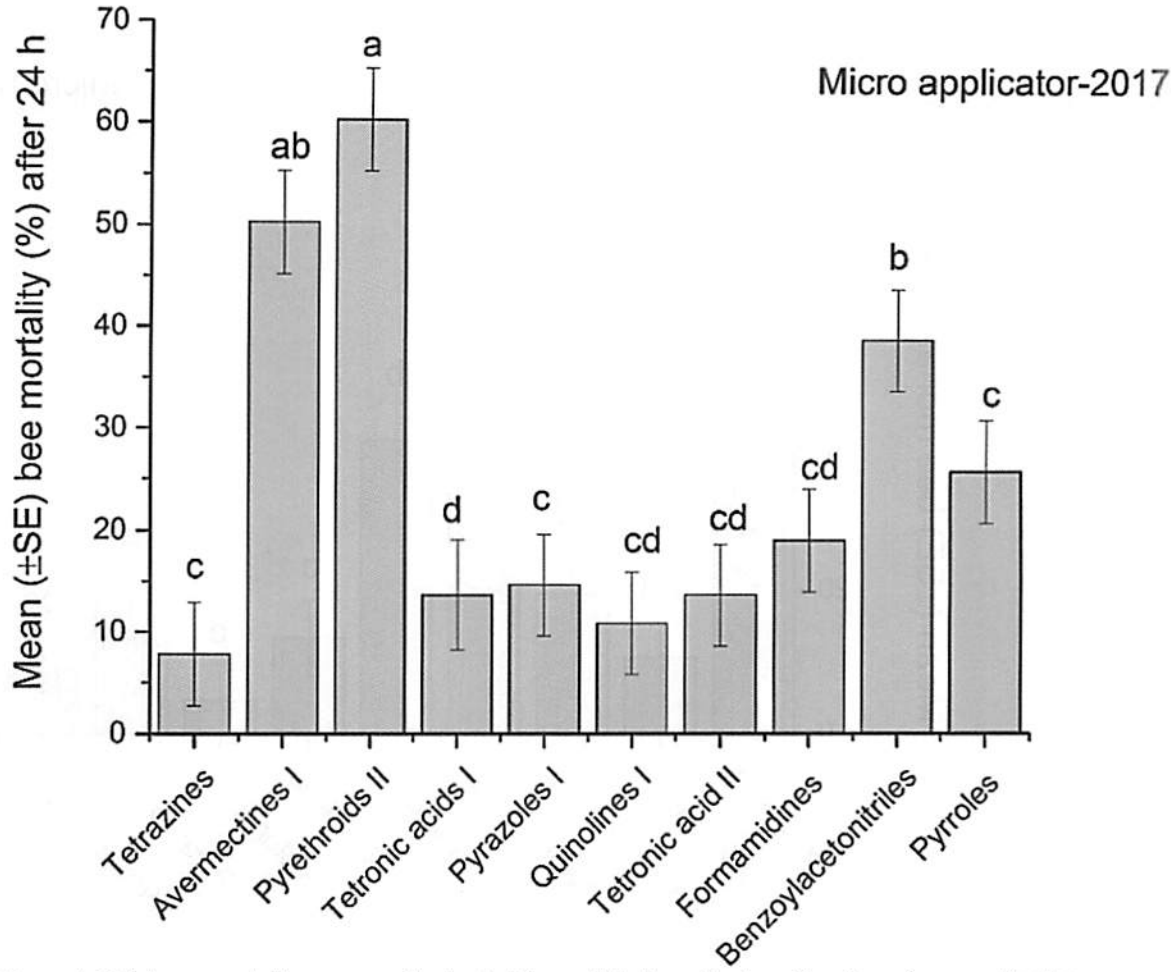

Figure A4.15. Mean (±SE) bee mortality exposed to tested formulated products using the micro applicator after 24 h. Means with the same letter among treatments are not significantly different (p<0.05) (2017).

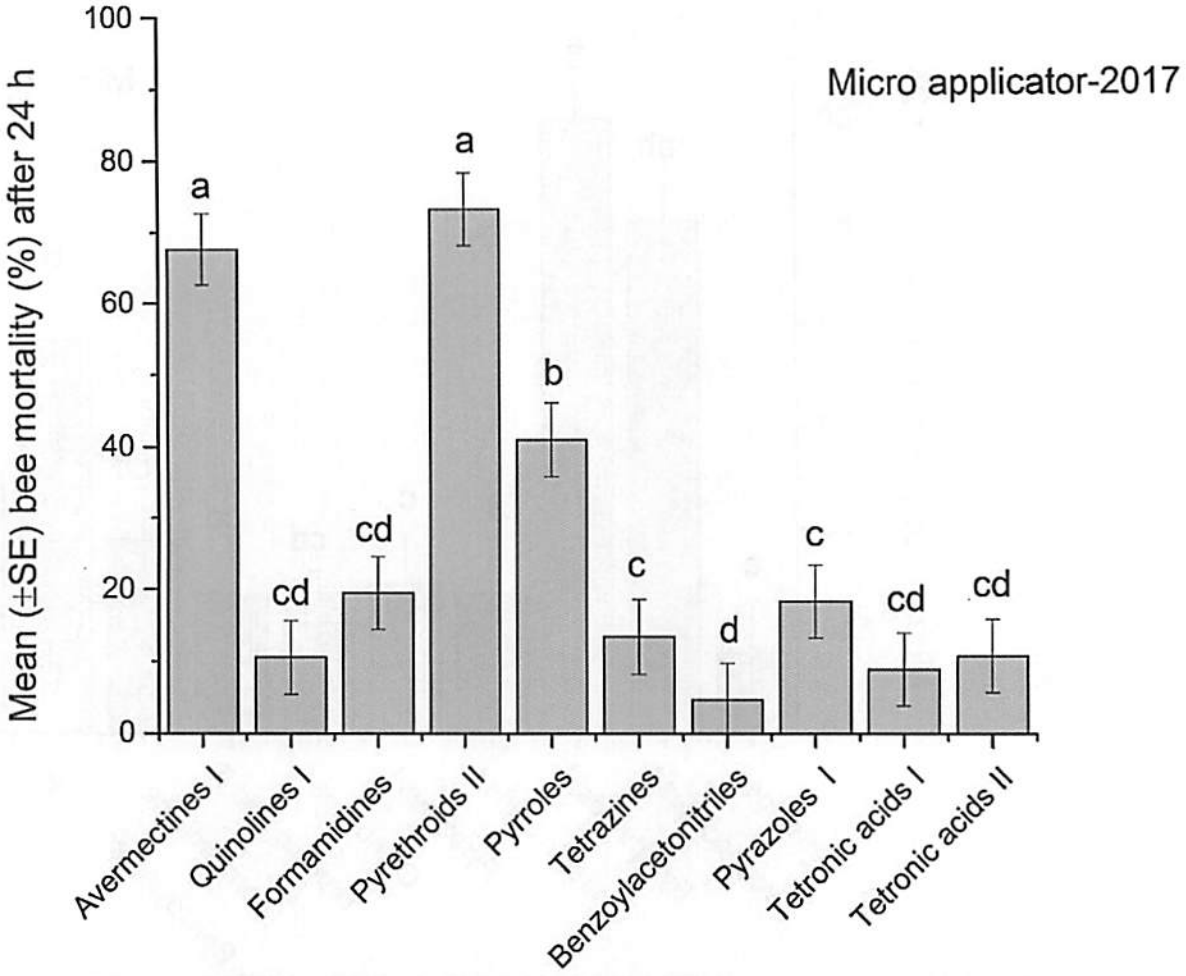

Figure A4.16. Mean (±SE) bee mortality exposed to tested active ingredients using the micro applicator after 24 h. Means with the same letter among treatments are not significantly different (p<0.05) (2017).

Table A4.4. Lethal dose values (LD_{50}) for 24 h responses of mites and bees to tested Formulated products (FP) and Active Ingredients (AI), 2017-2018.

FP/AI	Calss	LD50 mite	95%CL	95%CL	LD50 Bee	95%CL	95%CL
AI	Avermectine I	3.24E-07	3.91E-12	0.0000521	2.04E-10	6.78E-17	1.62E-07
FP	Avermectine I	.	.	.	1.27E-05	1.33E-06	7.21E-05
AI	Benzoylacetonitrile	4.03E-04	.	.	7.86E-06	2.92E-11	0.000865
FP	Benzoylacetonitrile	.	.	.	2.14E-25	.	.
AI	Formamidine	3.17E-08	1.78E-10	6.15E-07	2.20E-02	0.000846	8.93272
AI	Organochlorine I	9.64E-06	7.66E-07	0.0000493	0.00495	0.000629	0.02369
AI	Oxazoline	2.05E-87	.	.	1.04178	.	.
FP	Pyrazole I	.	.	.	5.99E-05	1.61E-06	0.00091
AI	Pyrazole I	8.04E-12	3.41E-16	3.09E-09	0.0003607	2.22E-06	3.26803
AI	Pyrazole II	0.0000171	3.63E-11	0.0003978	1.53E-07	4.18E-20	4.98E-05
AI	Pyrazole III	2.76E-06	3.81E-08	0.0000254	4.83E-15	.	.
AI	Pyrethroid I	7.88E-06	3.10E-07	0.0000537	0.0004271	2.07E-05	0.00352
AI	Pyrethroid II	1.96E-07	3.38E-21	0.0000199	6.04E-13	3.78E-40	2.73E-08
FP	Pyrethroid II	.	.	.	2.42E-10	1.84E-32	1.28E-06
AI	Pyrrole	6.23E-09	.	.	4.37E-08	4.65E-10	7.79E-07
FP	Pyrrole	.	.	.	2.24E-02	0.00403	0.07827
AI	Quinazoline	1.89E-06	3.75E-08	0.0000226	0.0001377	3.09E-06	0.00127
AI	Quinoline I	1031227	.	.	6.03E-05	6.97E-13	0.09094
FP	Quinoline II	.	.	.	1.69E-03	0.000191	0.01272
AI	Tetrazine	22891	.	.	5.28E-05	.	.
AI	Tetronic acid I	5.55E-01	0.01639	217982	6.00E-82	.	.
FP	Tetronic acid II	.	.	.	9.34E-07	1.34E-09	3.01E-05
AI	Tetronic acid II	3.39E-02	9.94E-05	3.72E+102	7.59E-02	0.00161	70477
AI	Thiazolidinone	0.0000863	9.21E-06	0.0005015	6.24E+41	.	.

Table A4.5. Evaluation of the efficacy of formulated products (FP) and their Active Ingredients (AI) against *Varroa* mites under field condition in 2017.

Treatment	AI mg/colony	Average daily mite mortality (±SE)
Formulated Products		
Tetronic acid I	250	0.01637±0.01906
	750	0.06753±0.01906
Pyrazole I	250	0.0863±0.01906
	750	0.2086±0.01906
Tetronic acid II	750	0.1131±0.01906
Benzoylacetonitrile	250	0.03597±0.01906
	750	0.0684±0.01906
	1000	0.02067±0.01906
Pyrrole	750	0.0188±0.01906
Active Ingredient		
Tetronic acid I	250	0.02593±0.01906
	750	0.09337±0.01906
Control	0	0.01367±0.02257

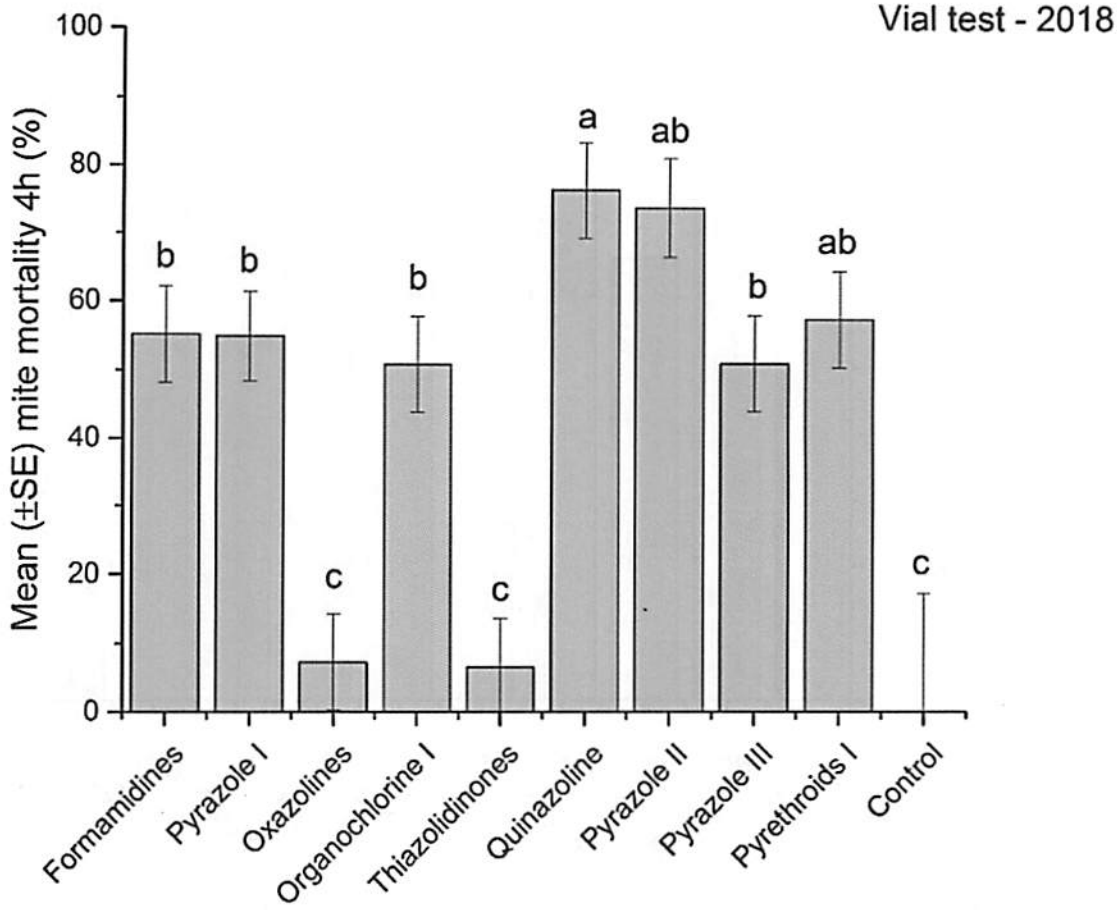

Figure A4.17. Mean (±SE) percentage mite mortality during 4 h exposed to tested active ingredients in glass vials under laboratory conditions. Means with the same letter among treatments are not significantly different ($p<0.05$) (2018).

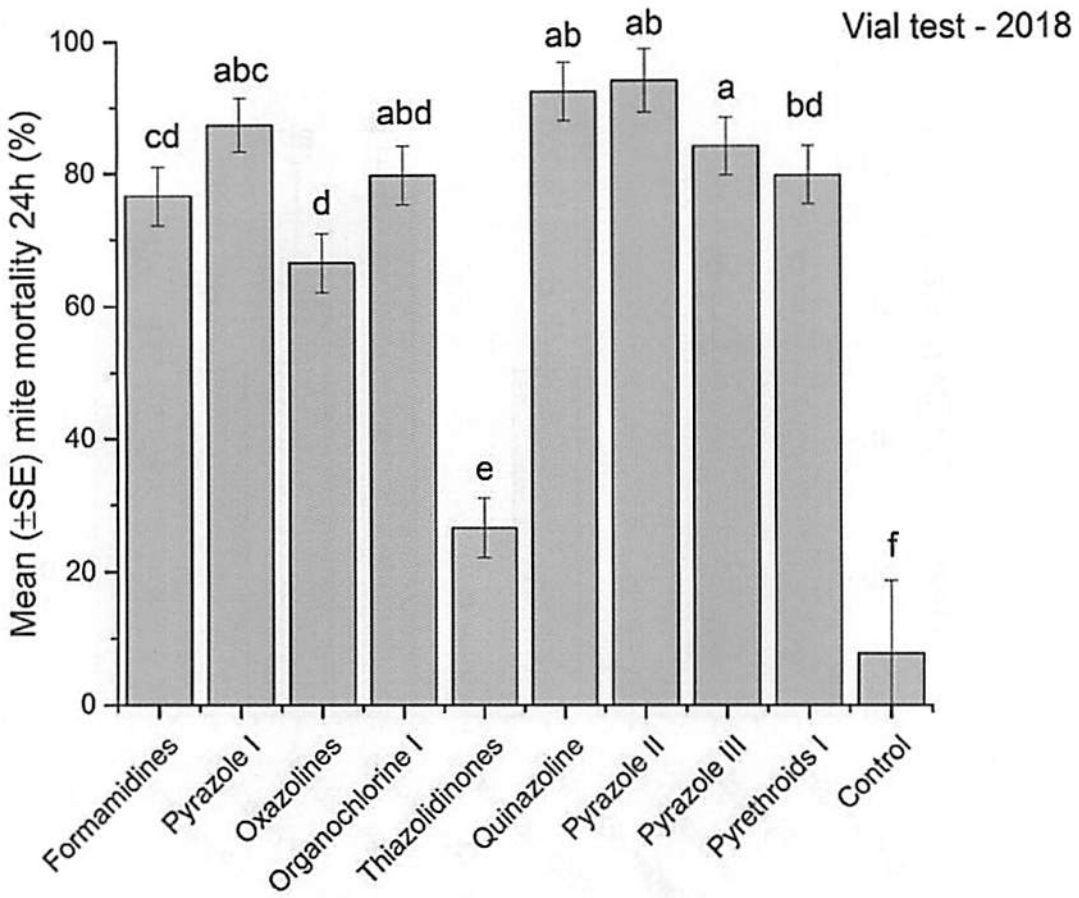

Figure A4.18. Mean (±SE) percentage mite mortality during 24 h exposed to tested active ingredients in glass vials under laboratory conditions. Means with the same letter among treatments are not significantly different ($p<0.05$) (2018).

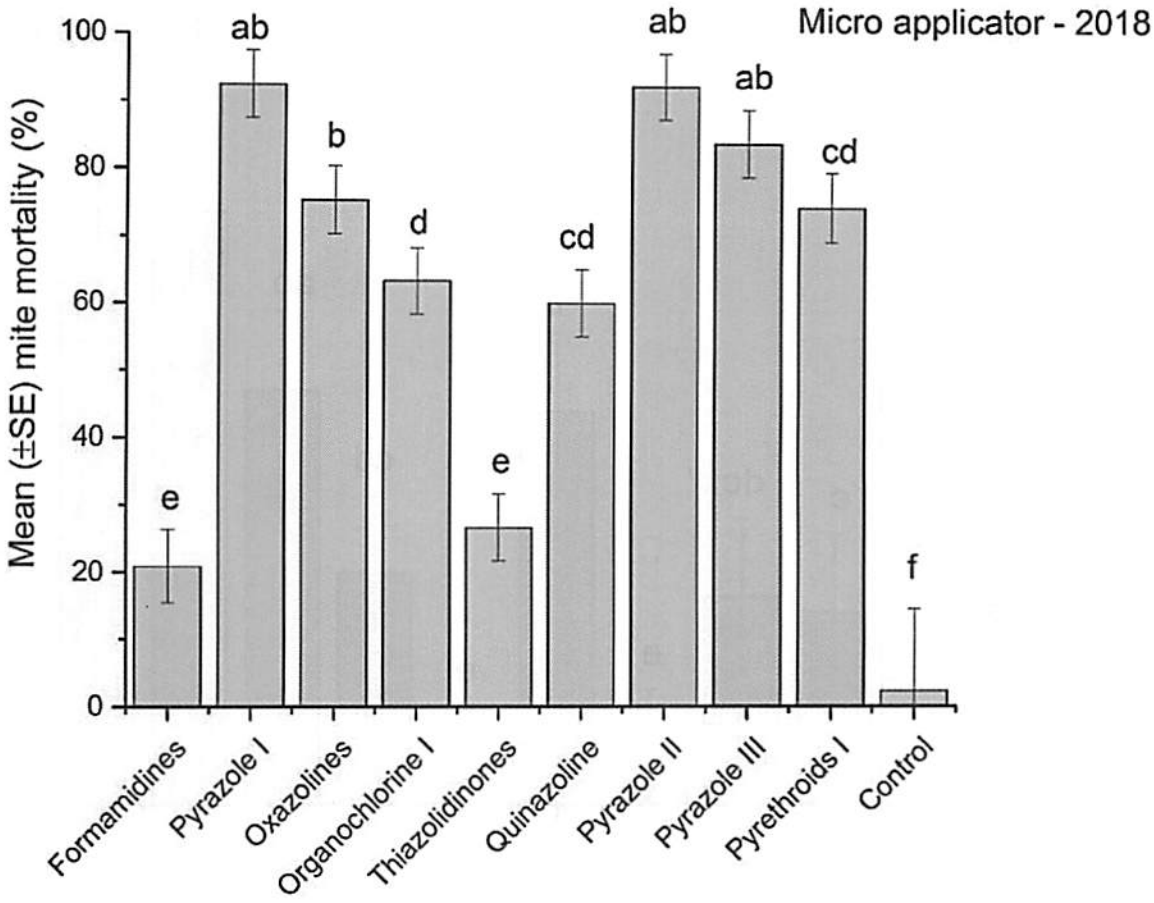

Figure A4.19. Mean (±SE) percentage mite mortality exposed to tested active ingredients using the micro applicator after 24 h. Means with the same letter among treatments are not significantly different ($p<0.05$) (2018).

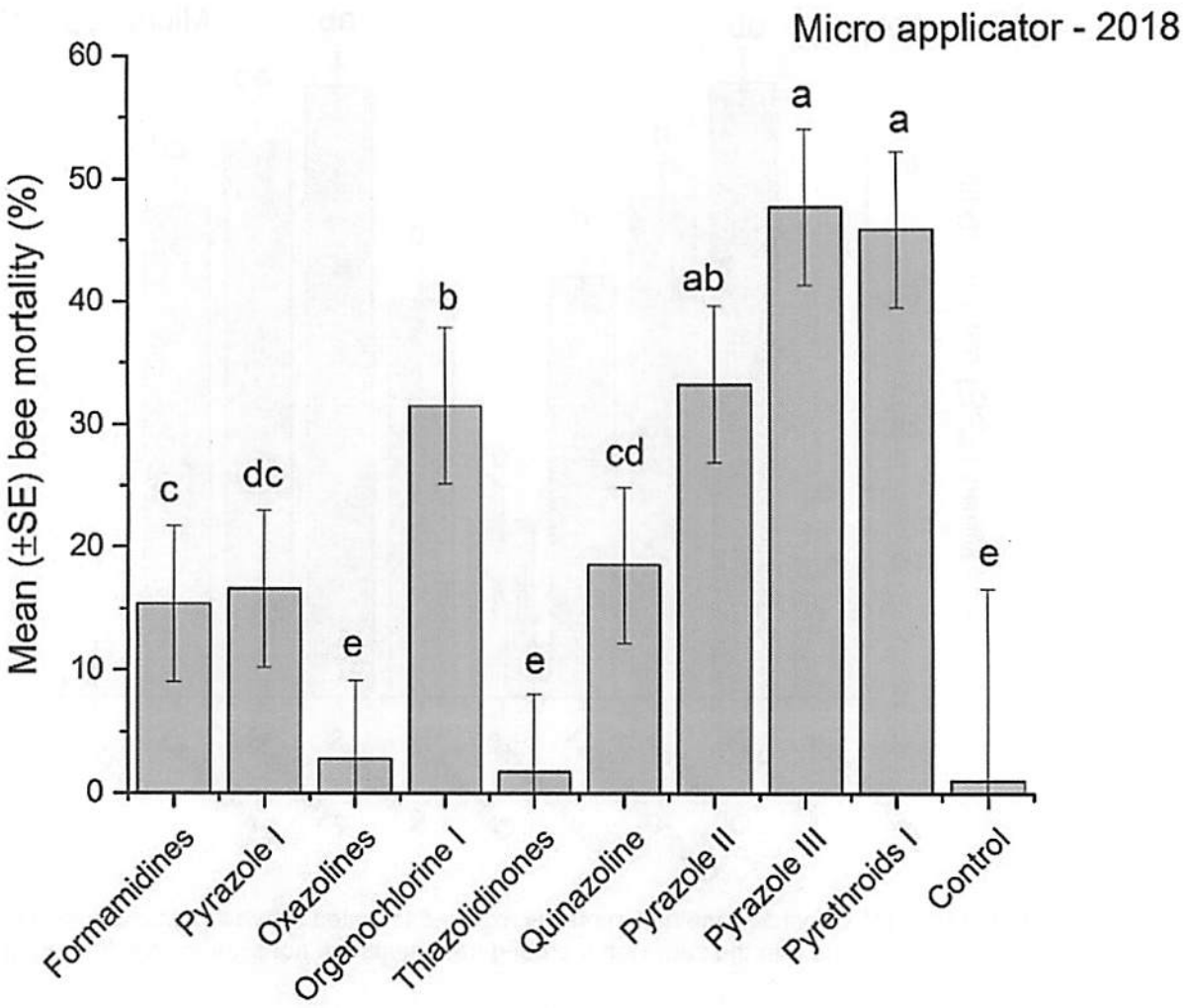

Figure A4.20. Mean (±SE) percentage adult bee mortality exposed to tested active ingredients using the micro applicator after 24 h. Means with the same letter among treatments are not significantly different ($p<0.05$) (2018).

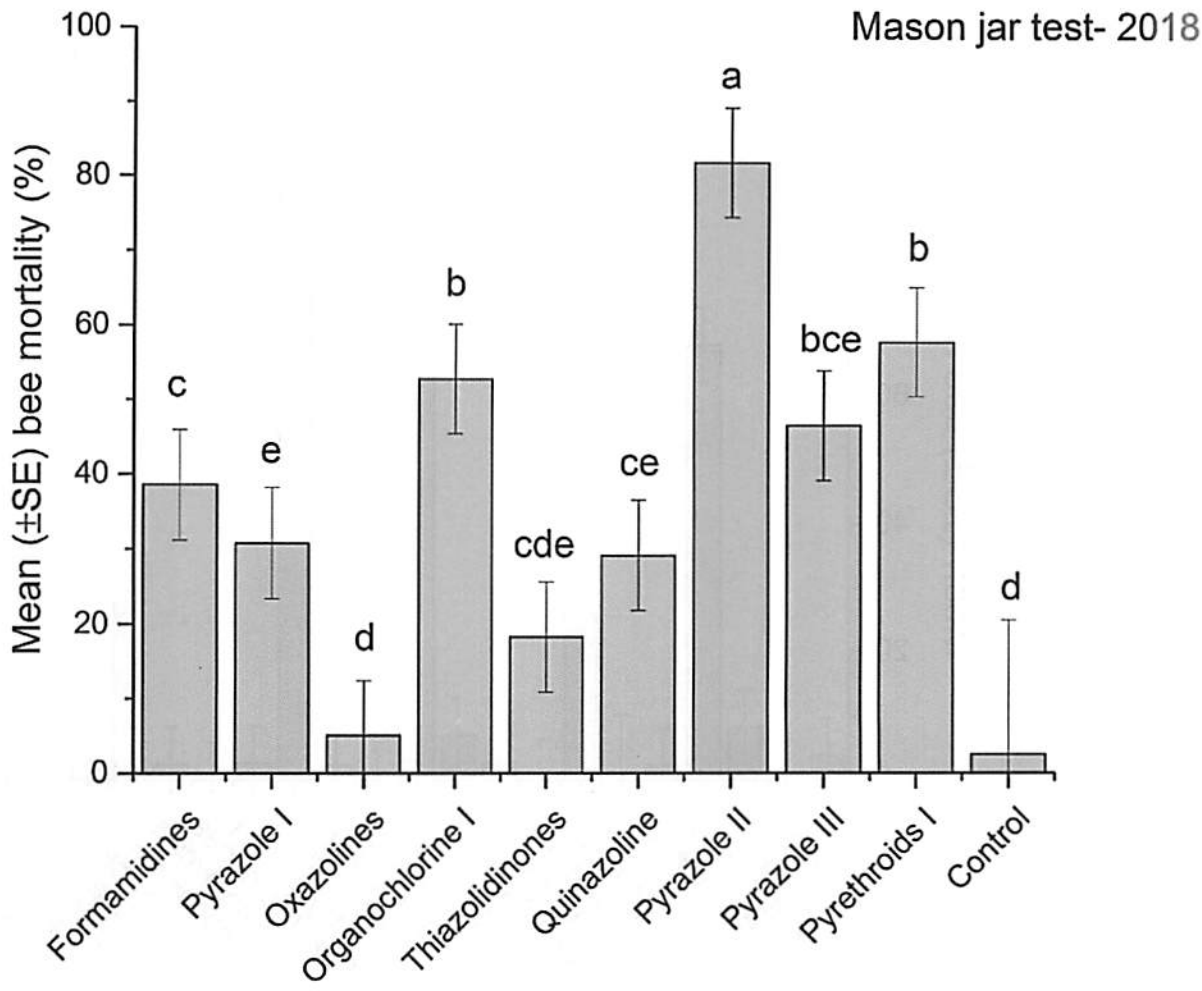

Figure A4.21. Mean (±SE) percentage bee mortality during 24 h exposed to tested active ingredients in mason jars under laboratory conditions. Means with the same letter among treatments are not significantly different ($p<0.05$) (2018).

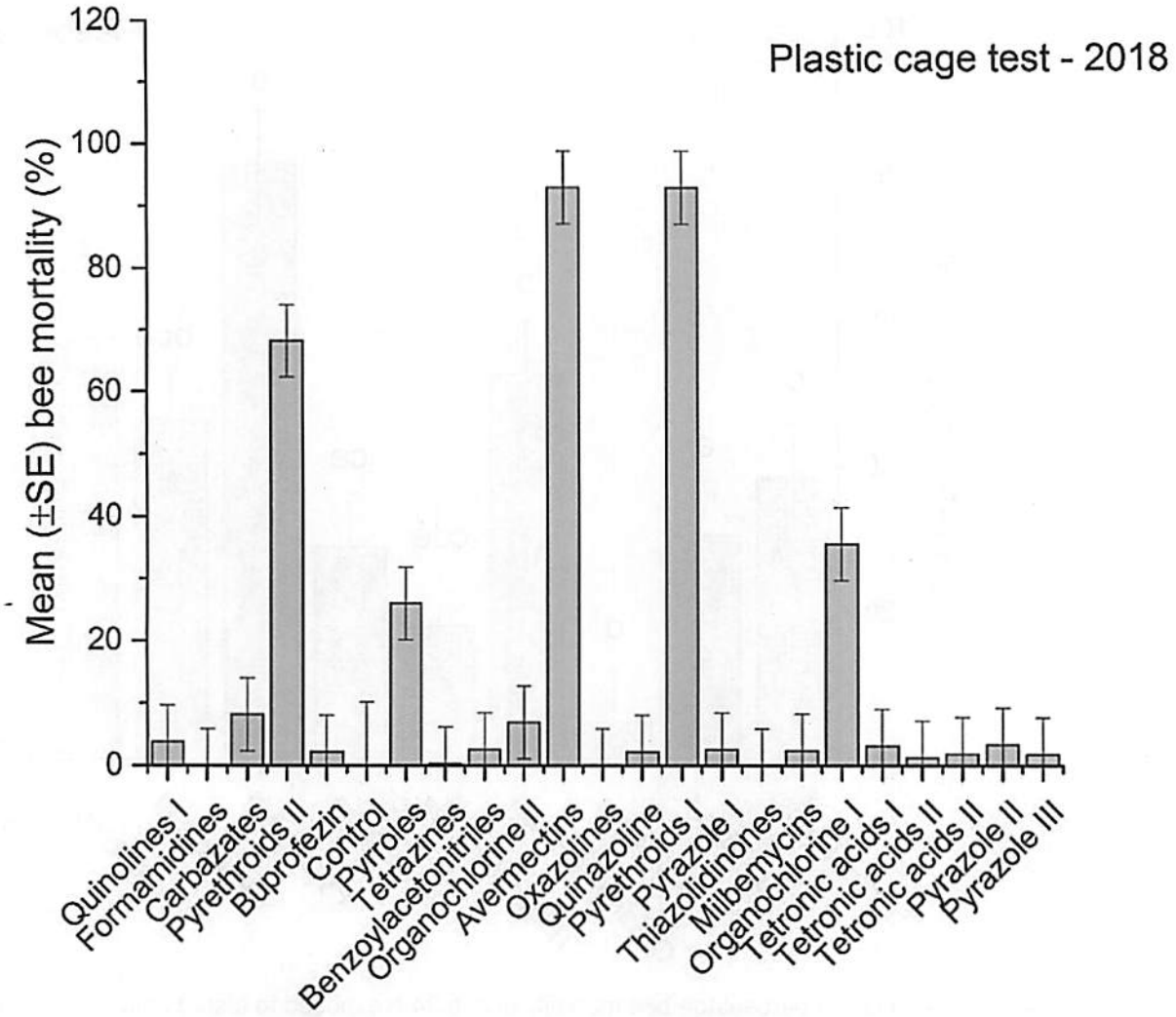

Figure A4.22. Mean (±SE) cumulative percentage adult bee mortality exposed to different active ingredients after 24 h in plastic cages (2018).

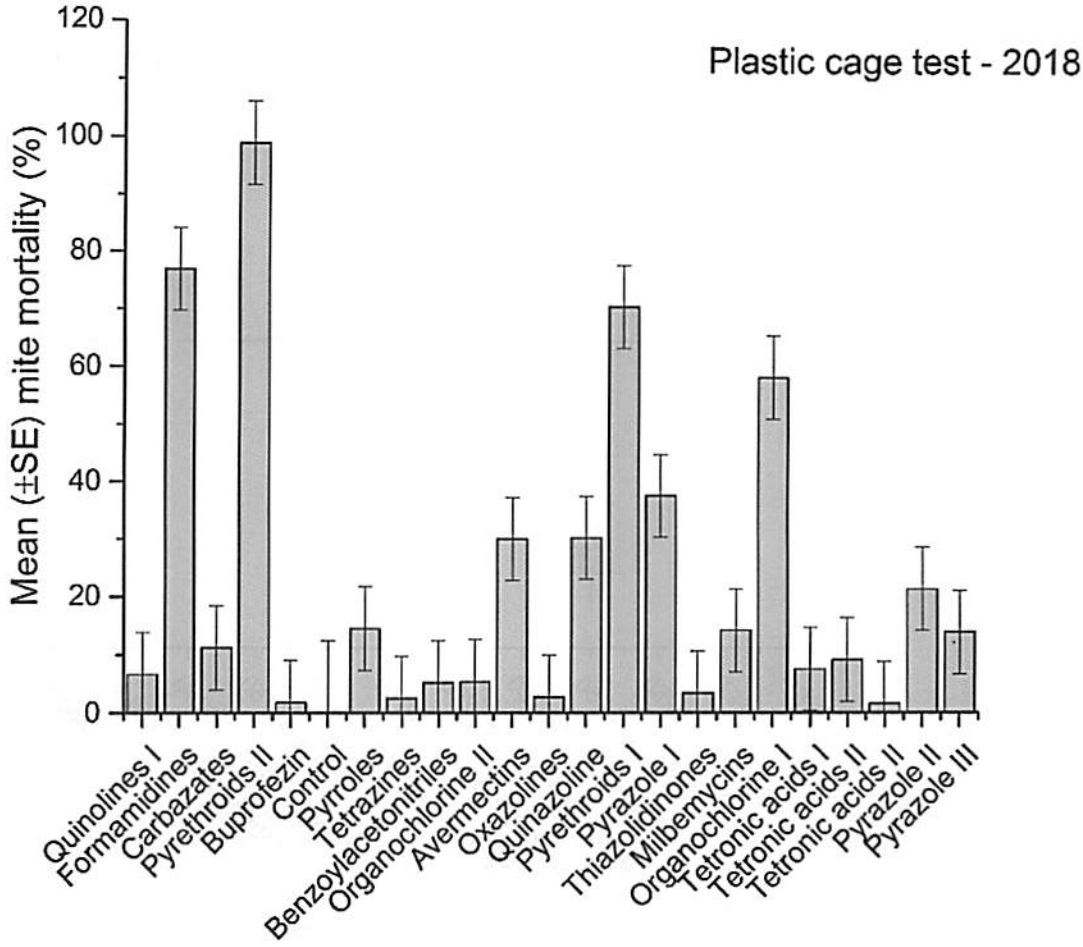

Figure A4.23. Mean (±SE) cumulative percentage mite mortality exposed to different active ingredients after 24 h in plastic cages (2018).

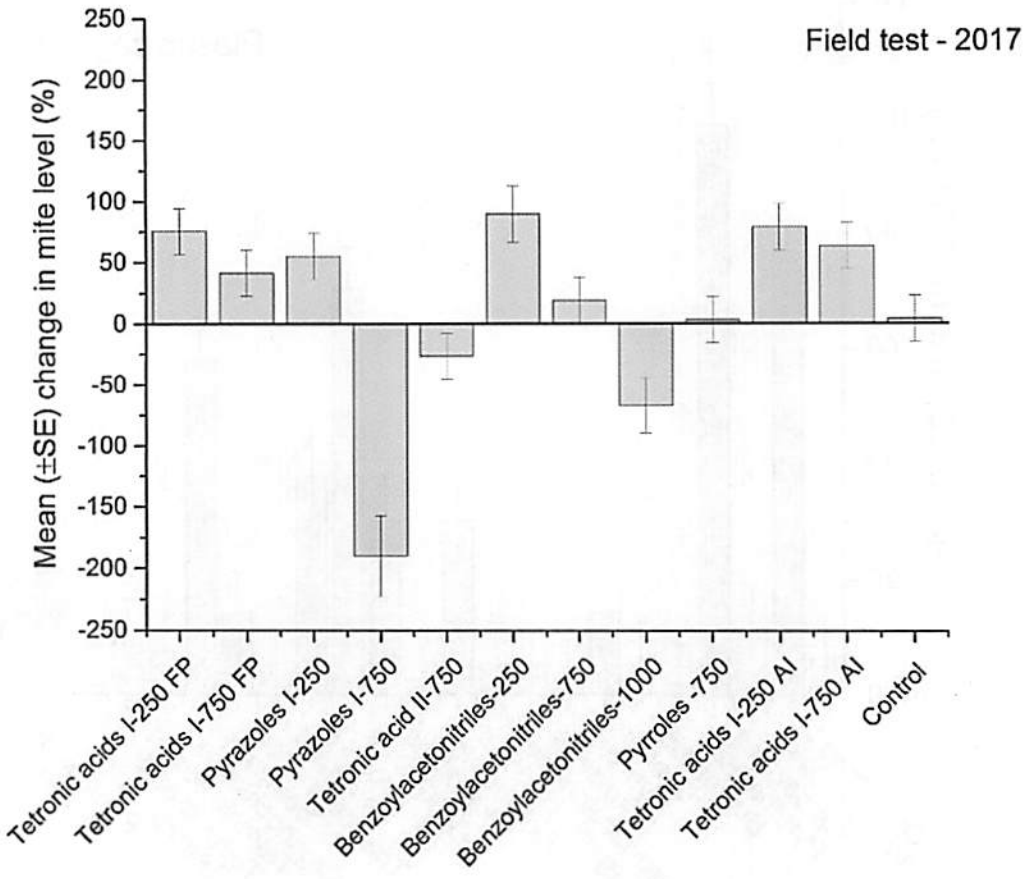

Figure A4.24. Mean (±SE) percentage changes in *Varroa* mite levels in colonies treated in the field study (2017). Results of analyzing data using a Before-After-Control-Impact (BACI) method showed that mite level significantly dropped in the pyrazole I (750 mg/colony), tetronic acid II (750 mg/colony) and benzoylacetonitrile (1000 mg/colony) treatment groups over time ($P < 0.05$), however, mite level increased in other treatment groups during the experiment.

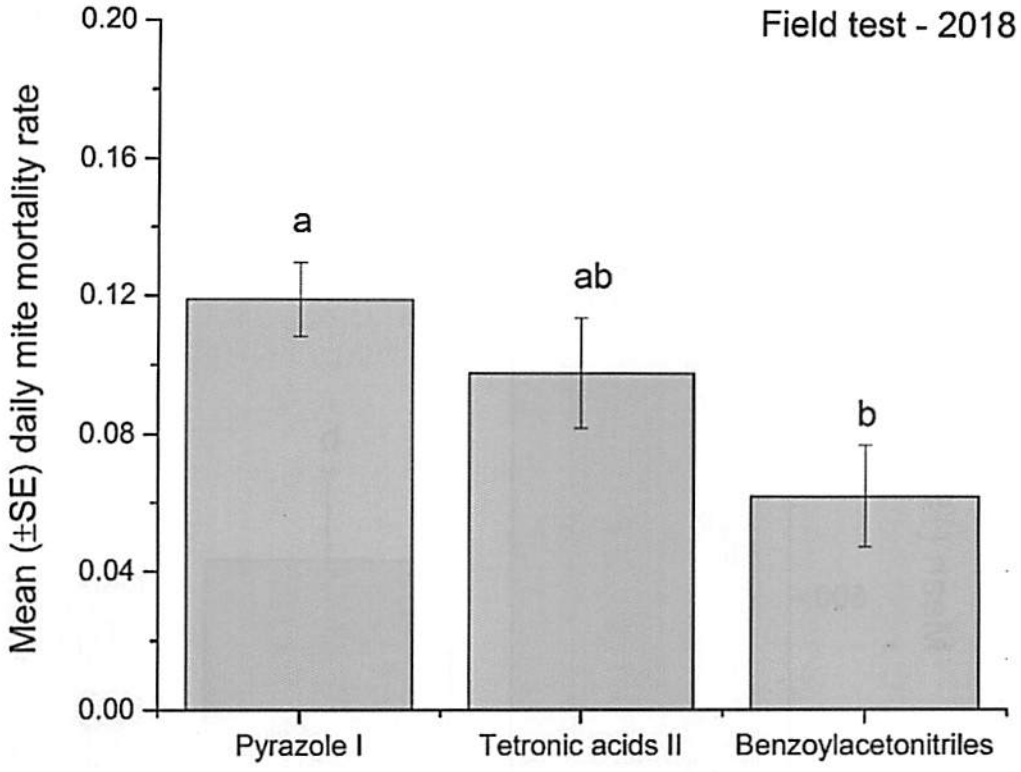

Figure A4.25. Mean (±SE) cumulative daily *Varroa* mite mortality when honey bee colonies were exposed to different doses (500, 1000, and 1500 mg/ colony) of pyrazole I, tetronic acid II, or benzoylacetonitrile in field test. Each treatment included 9 colonies. Means with the same letter among treatments are not significantly different ($p<0.05$) (2018).

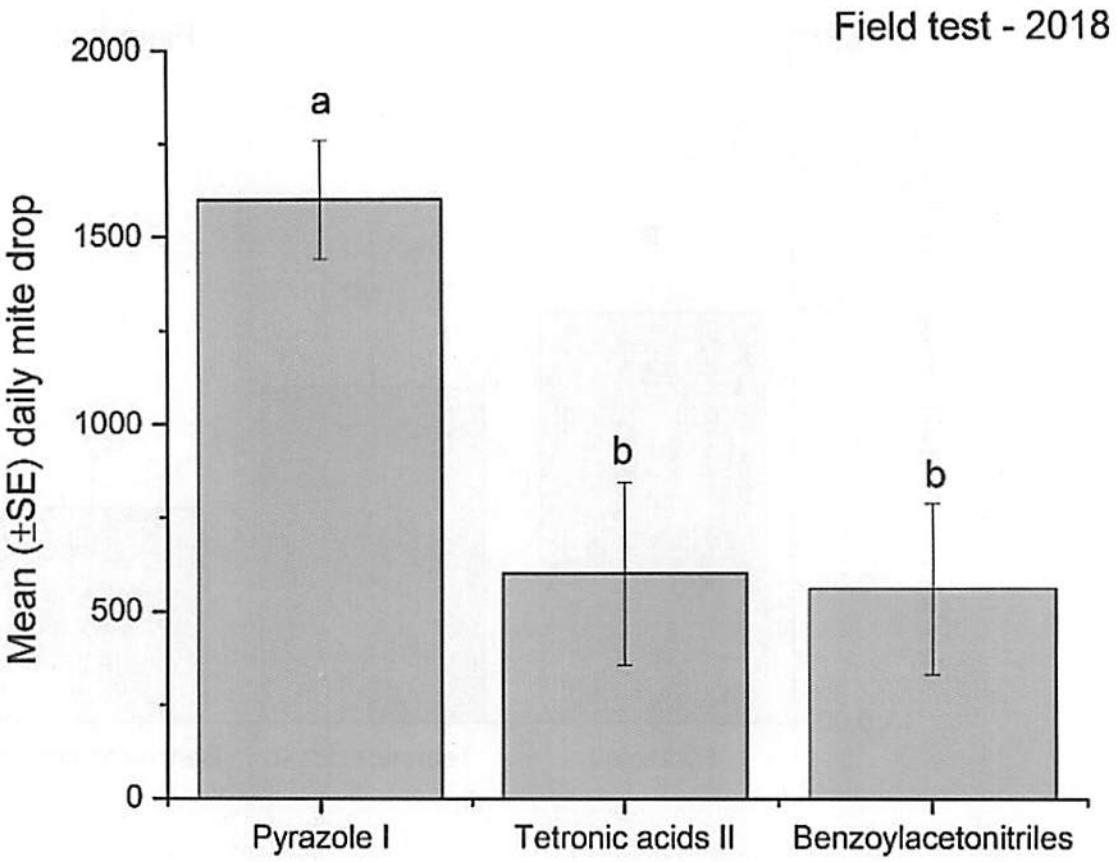

Figure A4.26. Mean (±SE) cumulative daily mite drop on sticky boards in treated colonies with different doses (500, 1000, and 1500 mg/ colony) of pyrazole I, tetronic acid II, or benzoylacetonitrile in the field test. Each treatment included 9 colonies. Means with the same letter are not significantly different ($p<0.05$) (2018).

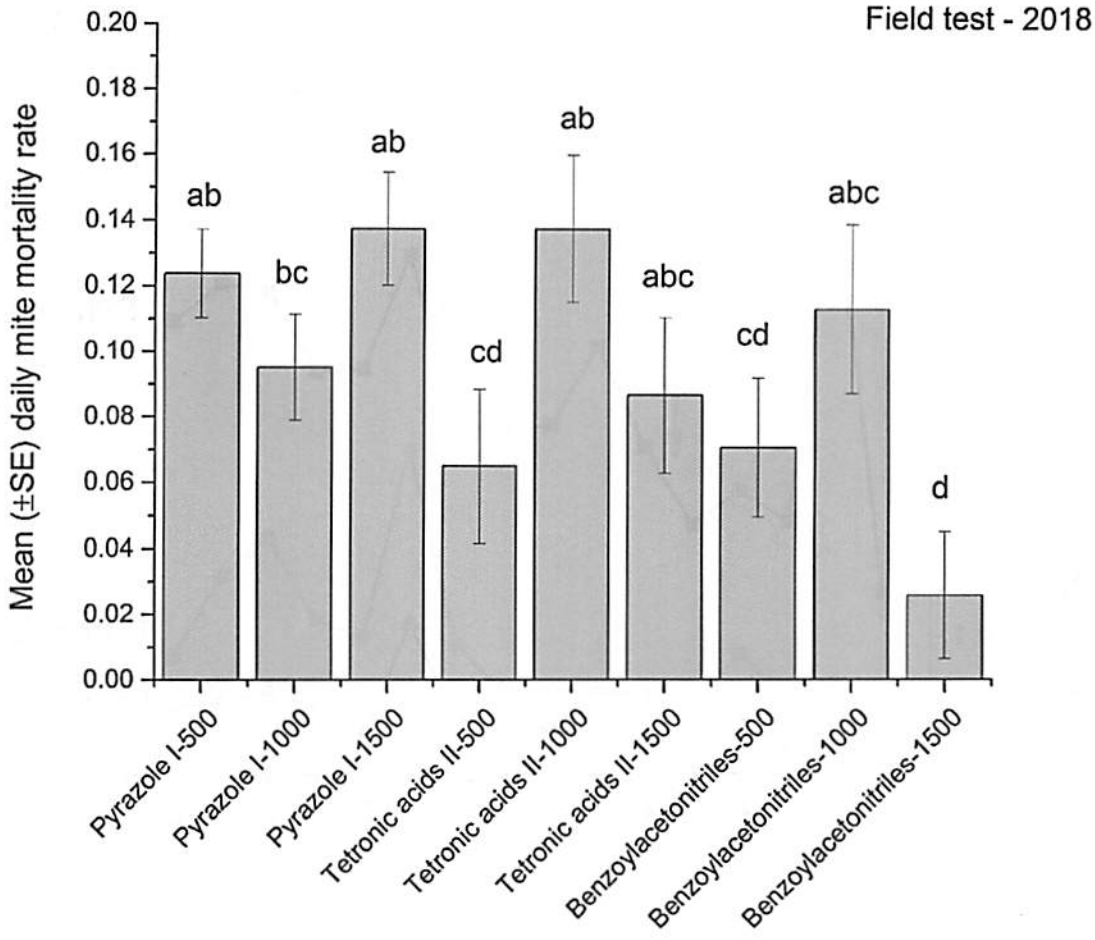

Figure A4.27. Mean (±SE) cumulative daily mite mortality in treated colonies with different doses (500, 1000, and 1500 mg/ colony) of pyrazole I, tetronic acid II, and benzoylacetonitrile in the field test. Each dose included 3 colonies. Means with the same letter among treatments are not significantly different ($p<0.05$) (2018).

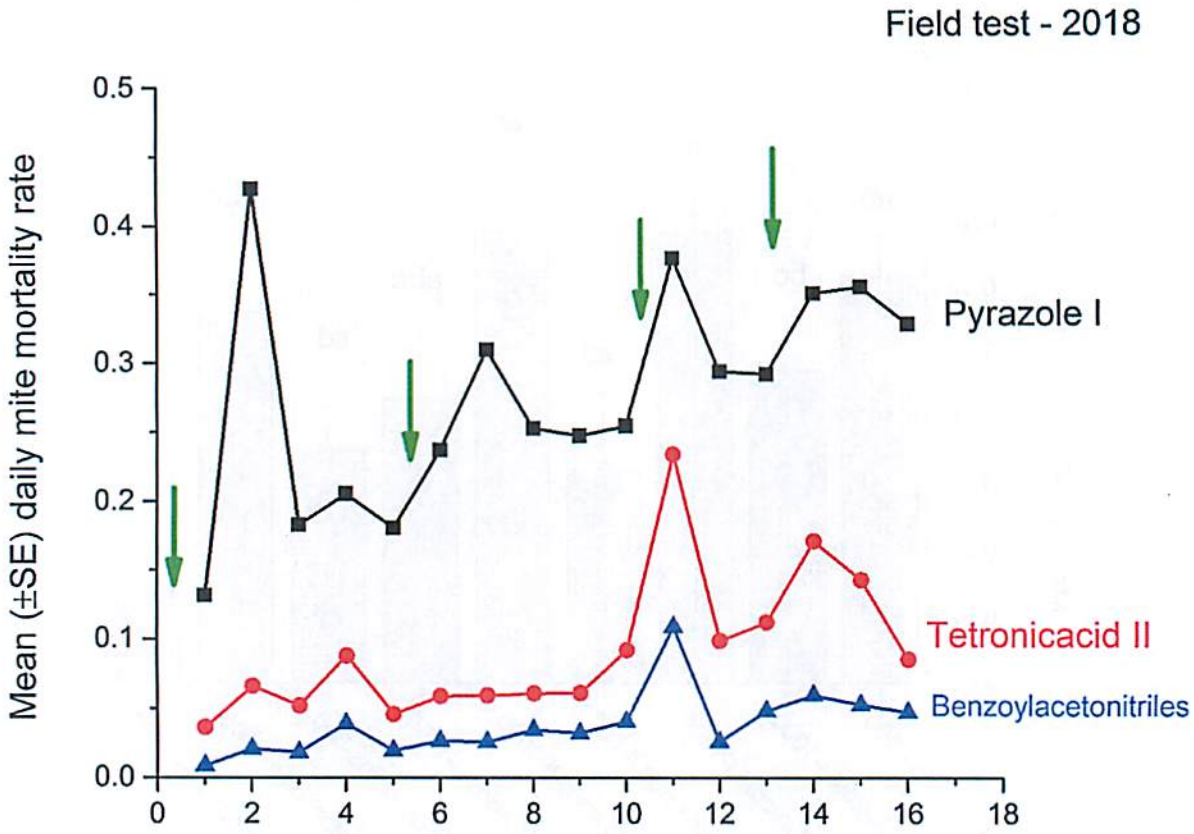

Figure A4.28. Mean (±SE) cumulative daily mite mortality in treated colonies with different doses (500, 1000, and 1500 mg/ colony) of pyrazole I, tetronic acid II, and benzoylacetonitrile in field test over treatment periods. Each treatment included 9 colonies. The X-axis indicates the time point of sampling (Sep 5 – Oct 1, 2018). Arrows indicate time points of treatments (2018).

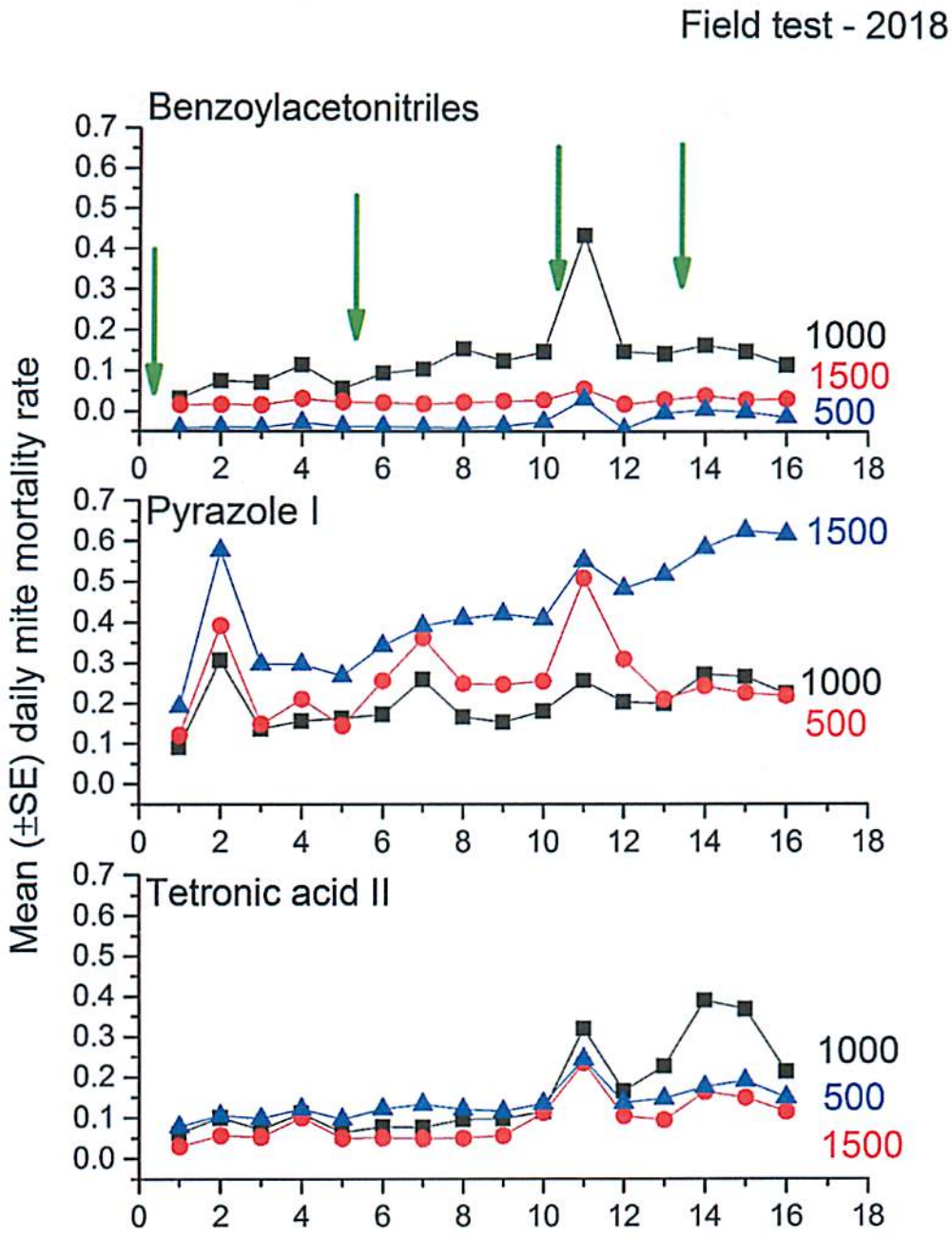

Figure A4.29. Mean (±SE) daily mite mortality in treated colonies with different doses (500, 1000, and 1500 mg/ colony) of pyrazole I, tetronic acid II, and benzoylacetonitrile in field test over treatment periods. Each dose included 3 colonies. The X-axis indicates the time point of sampling (Sep 5 – Oct 1, 2018). Arrows indicate time points of treatments (2018).

Appendix 5 Objective 2 (a-c) Developing new treatments for *Nosema*

Table A5.1- Antimicrobials tested in laboratory and field for treatment of honey bees against *Nosema* spp.

Product	2017	2018	
	Lab - %	Lab - %	Field - %
Mebendazole	0.1/0.01/0.001/0.0001/0.00001	1 / 0.5 / 0.25 / 0.125	
Metronidazole	0.1/0.01/0.001/0.0001/0.00001		
Quinine	0.1/0.01/0.001/0.0001/0.00001		
Genistein	0.1/0.01/0.001/0.0001/0.00001		
Artemisinin	0.1/0.01/0.001/0.0001/0.00001	1 / 0.5 / 0.25 / 0.125	0.1/0.01/0.001/0.0001
Amprolium	0.1/0.01/0.001/0.0001/0.00001		
Nitazoxanide		0.1/0.01/0.001/0.0001/0.00001	
Ornidazole		0.1/0.01/0.001/0.0001/0.00001	
Nitrofurazone		0.1/0.01/0.001/0.0001/0.00001	
Fenbendazole		0.1/0.01/0.001/0.0001/0.00001	
Curcumin		0.1/0.01/0.001/0.0001/0.00001	
Fumagillin	0.1/0.01/0.001/0.0001/0.00001	1 / 0.5 / 0.25 / 0.125	0.0042
Dimethoate	0.033		
Positive	0	0	0
Negative	0	0	

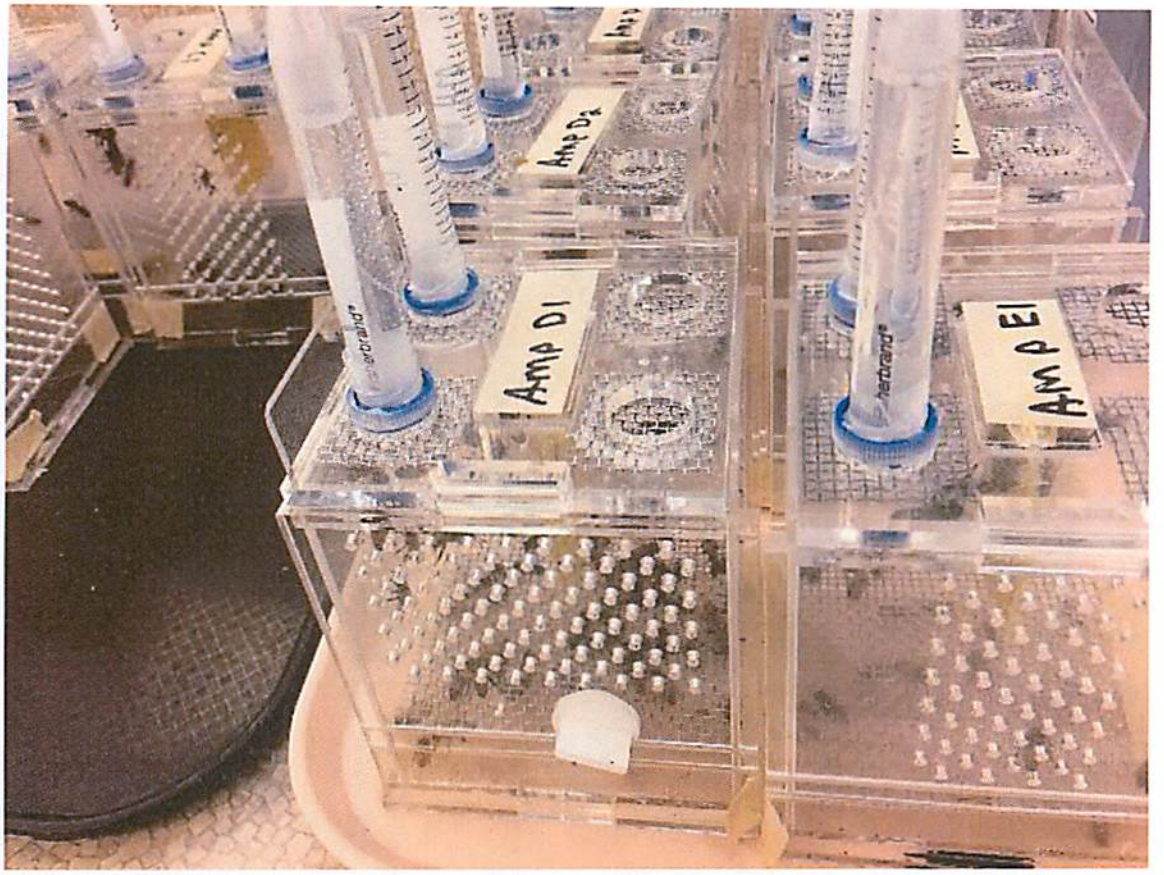

Figure A5.1: A group of 100-120 newly emerged worker bees were confined in the bioassay Plexiglas® cages and fed water and 50% syrup contain candidate compounds using gravity tube feeders (2017-2018).

Figure A5.2 *Nosema*-infected small colonies (nucs) were sprayed with fumagillin or one of four concentrations of artemisinin (2018).

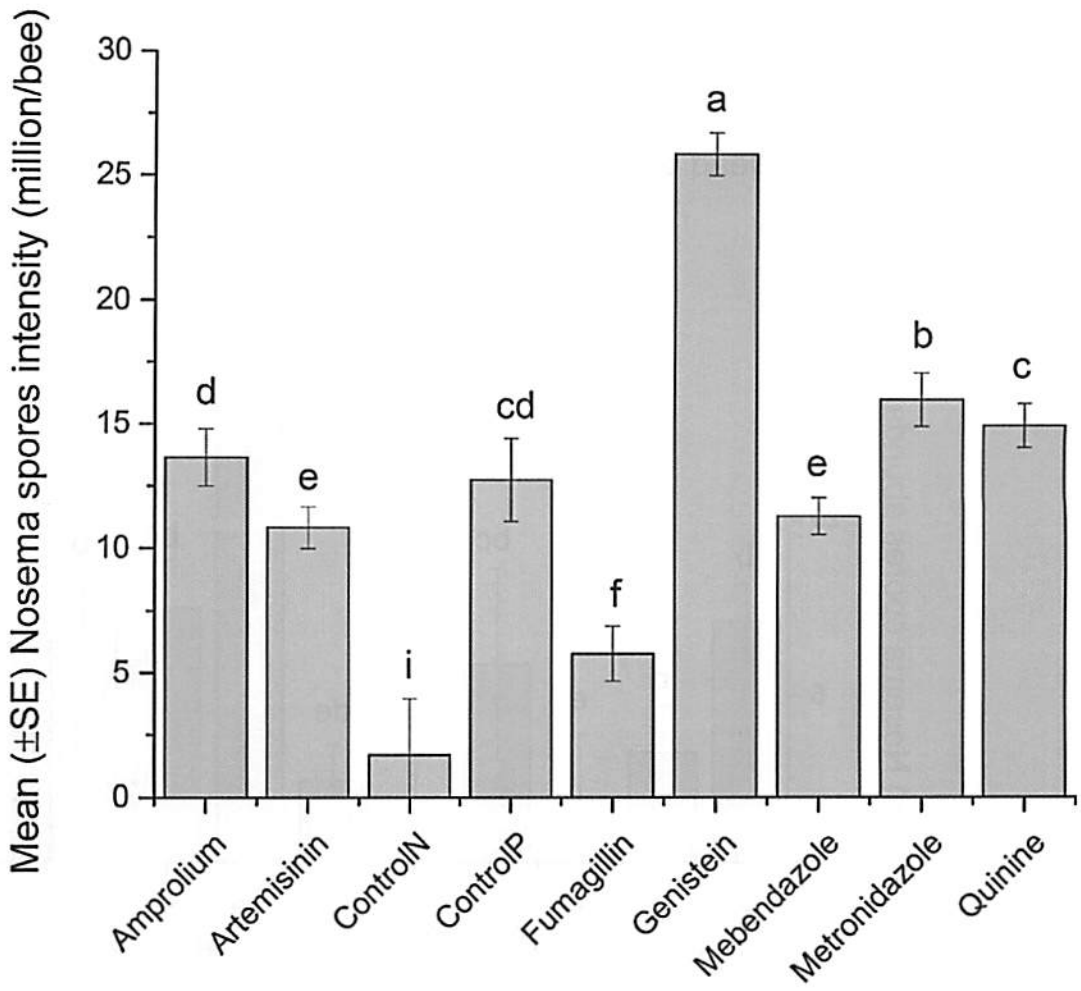

Figure A5.3. Mean (±SE) *Nosema* spore intensity (million per infected-bee) in live bees that were collected at day 20 post-inoculation in different treatments (2017). Means of treatments with the same letter are not significantly different ($p< 0.05$).

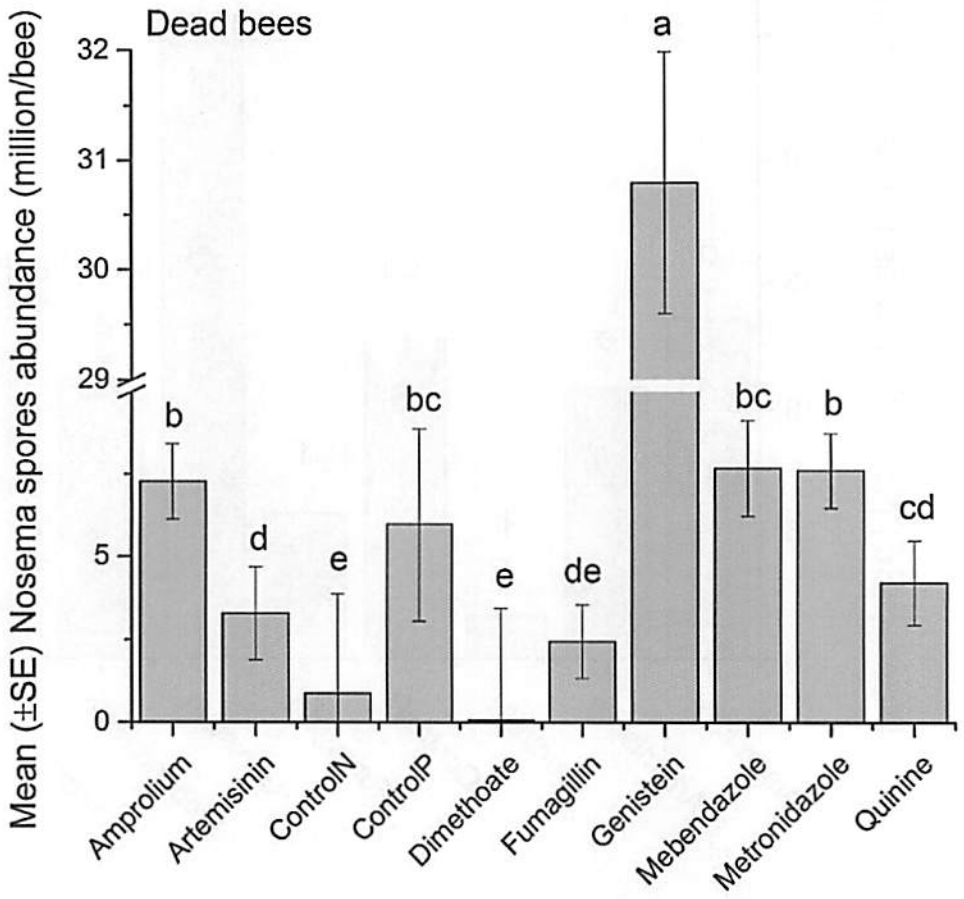

Figure A5.4. Mean (±SE) *Nosema* spore abundance (million per bee) in dead bees across treatments (2017). Means of treatments with the same letter are not significantly different ($p < 0.05$).

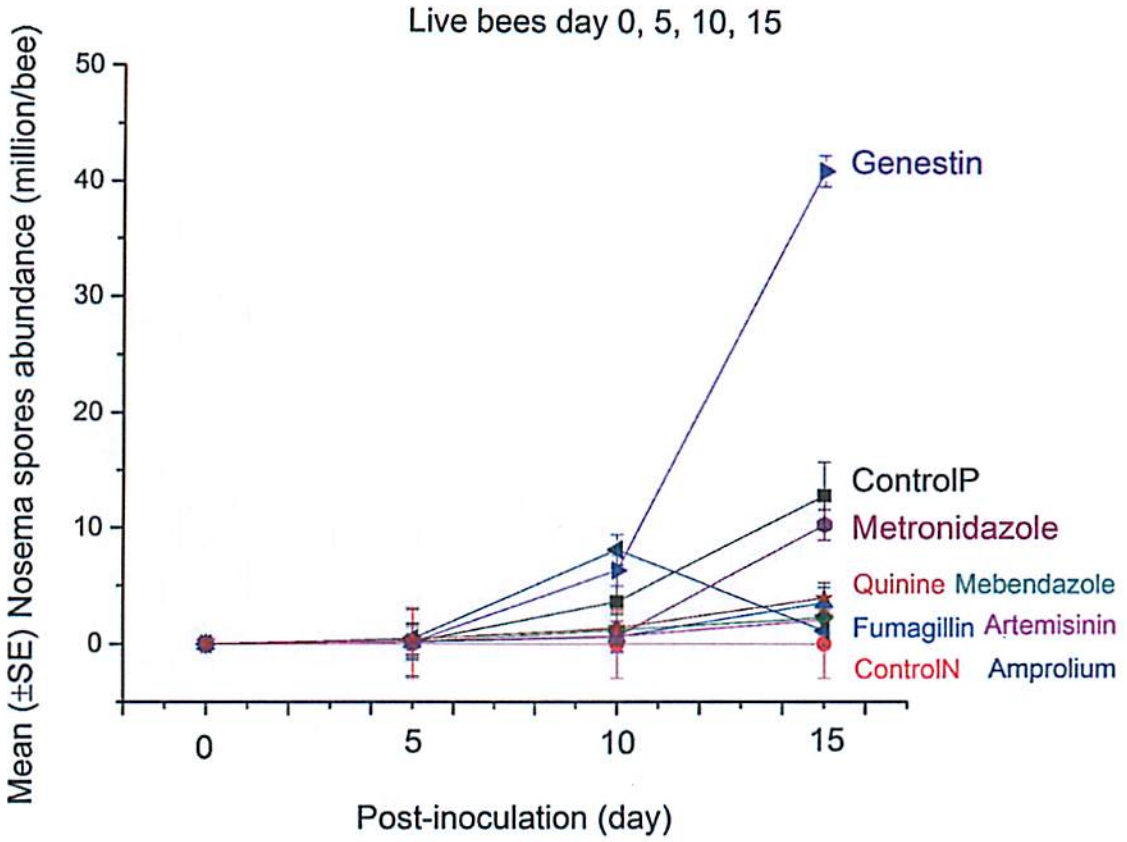

Figure A5.5. Mean (±SE) *Nosema* spore abundance (million per bee) in live bees collected at days 0, 5, 10 and 15 post-inoculation (2017).

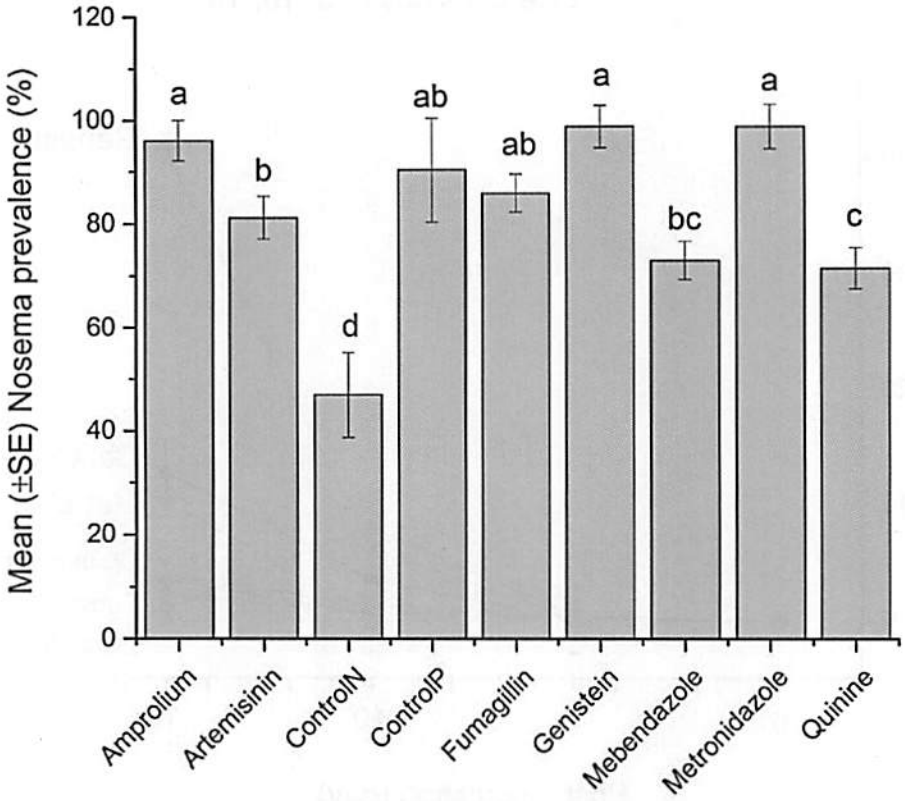

Figure A5.6: Mean (±SE) *Nosema* prevalence (%) in live bees that were collected at day 20 post-inoculation in different treatments (2017). Means of treatments with the same letter are not significantly different ($p<$ 0.05).

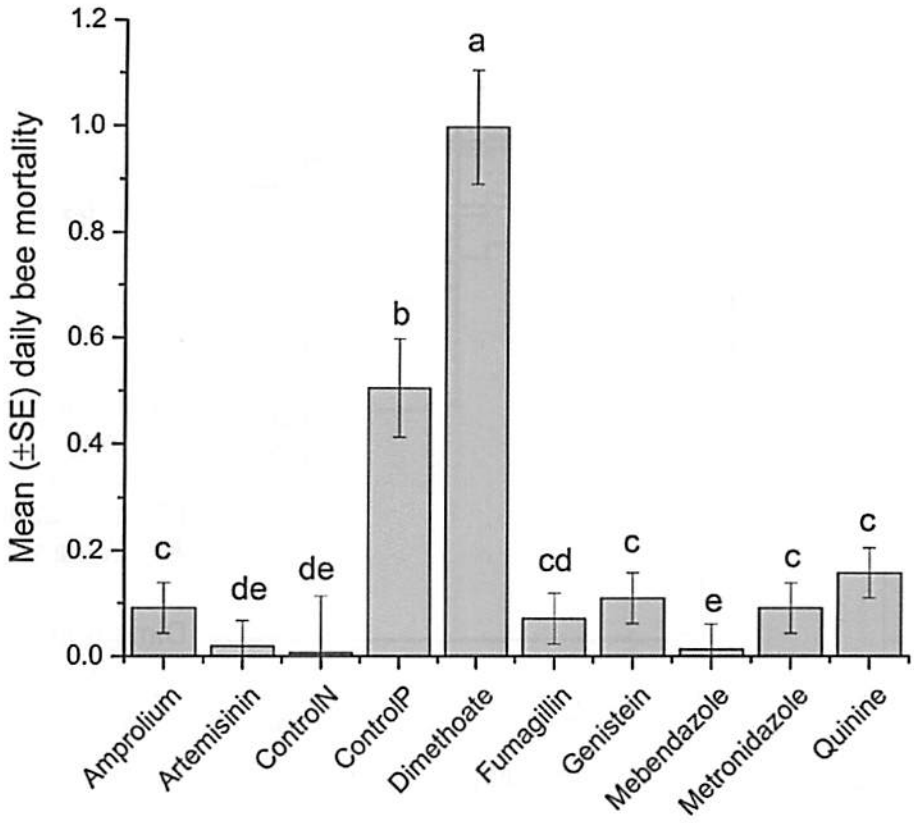

Figure A5.7. Mean (±SE) cumulative daily mortality of worker bees in different treatments collected post-inoculation (2017). Means of treatments with the same letter are not significantly different ($p< 0.05$).

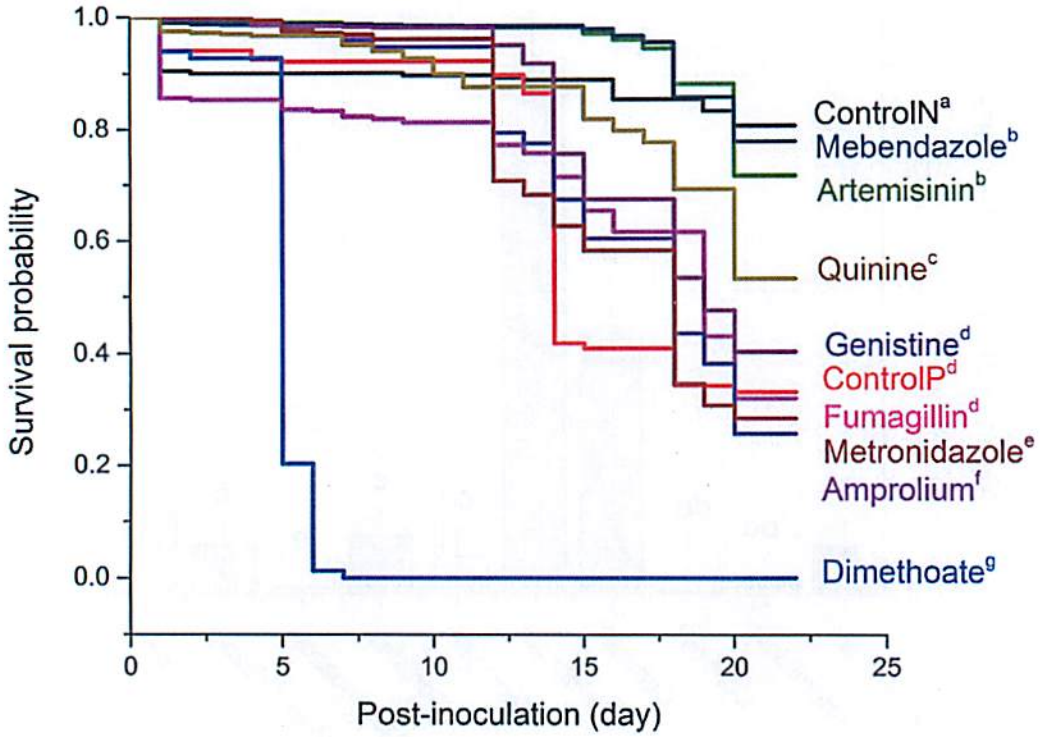

Figure A5.8. Survival plot of worker bees across experimental treatment groups (2017). Treatment groups followed by the same letter are not significantly different ($p < 0.05$).

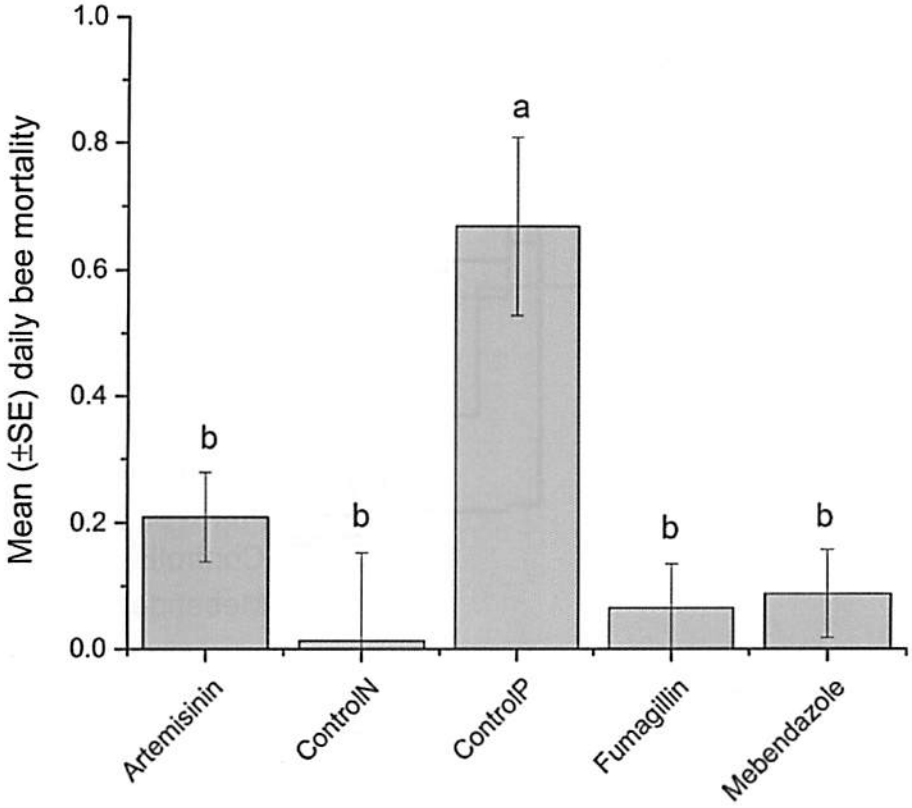

Figure A5.9. Mean (±SE) cumulative daily mortality of worker bees in different treatments collected post-inoculation (2018). Means of treatments with the same letter are not significantly different ($p< 0.05$).

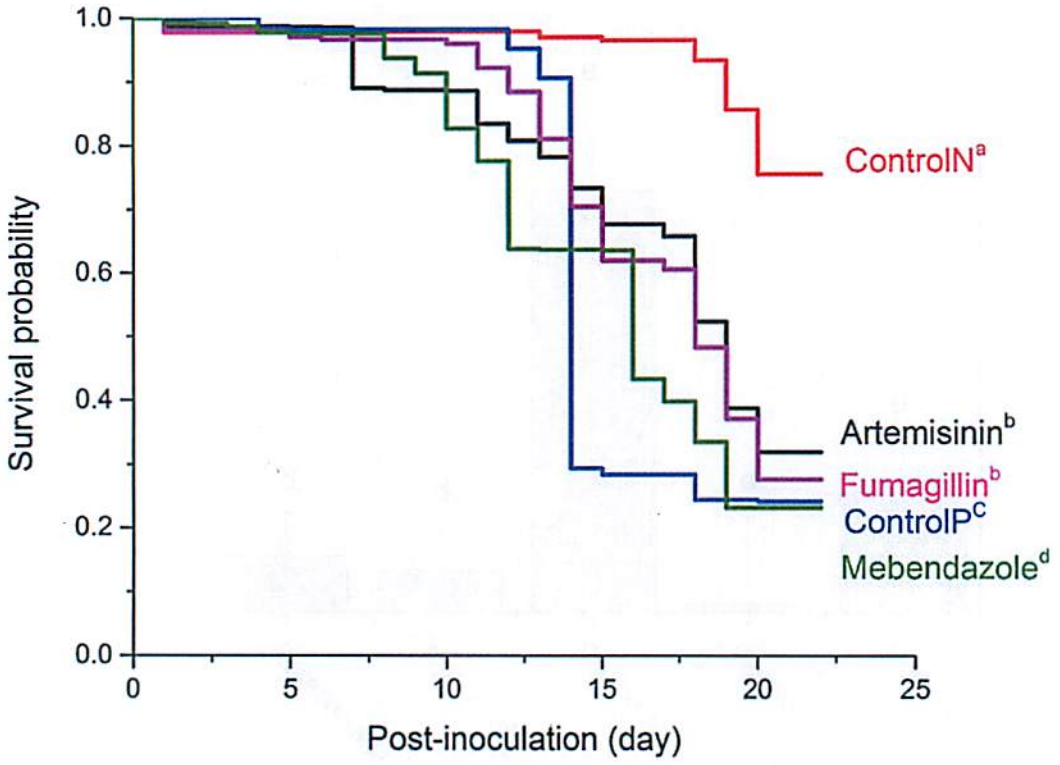

Figure A5.10. Survival plot of worker bees across experimental treatment groups (2018). Treatments followed by the same letter are not significantly different ($p< 0.05$).

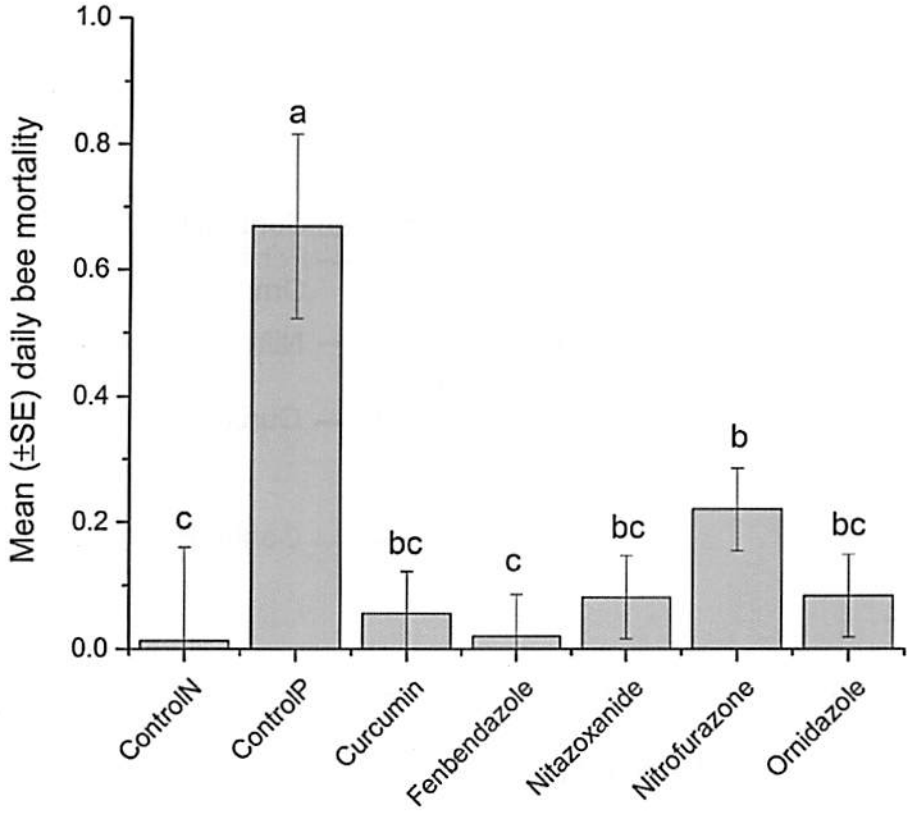

Figure A5.11. Mean (±SE) cumulative daily mortality of worker bees in different treatments collected post-inoculation (2018). Means of treatments with the same letter are not significantly different ($p< 0.05$).

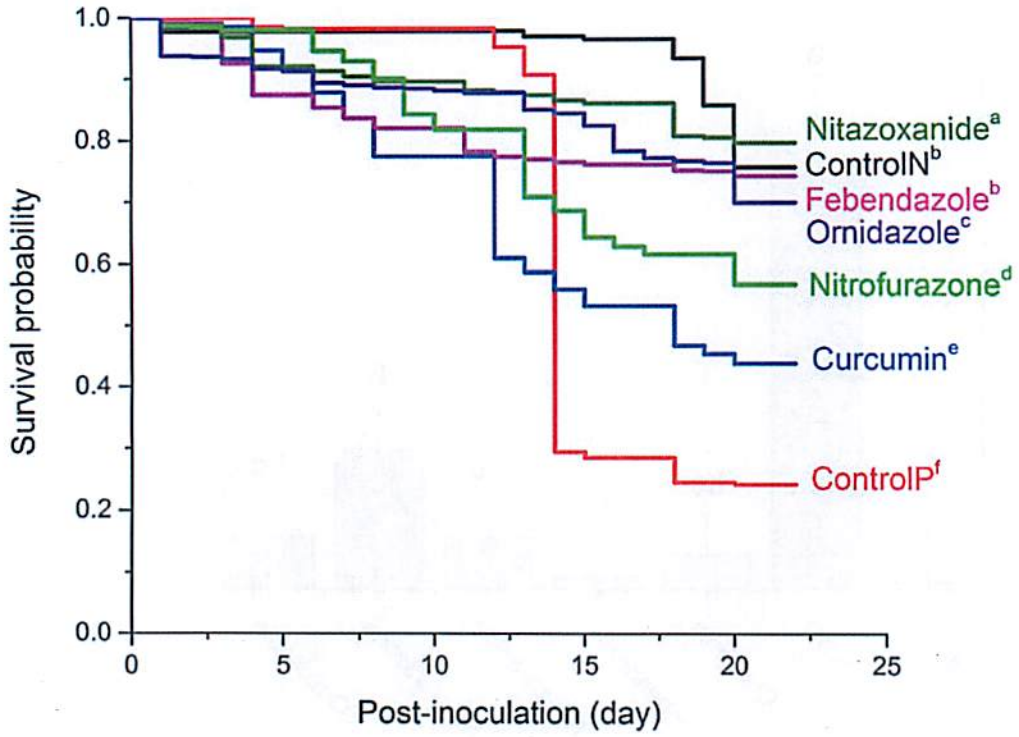

Figure A5.12. Survival plot of worker bees across experimental treatment groups (2018). Treatments followed by the same letter are not significantly different ($p< 0.05$).

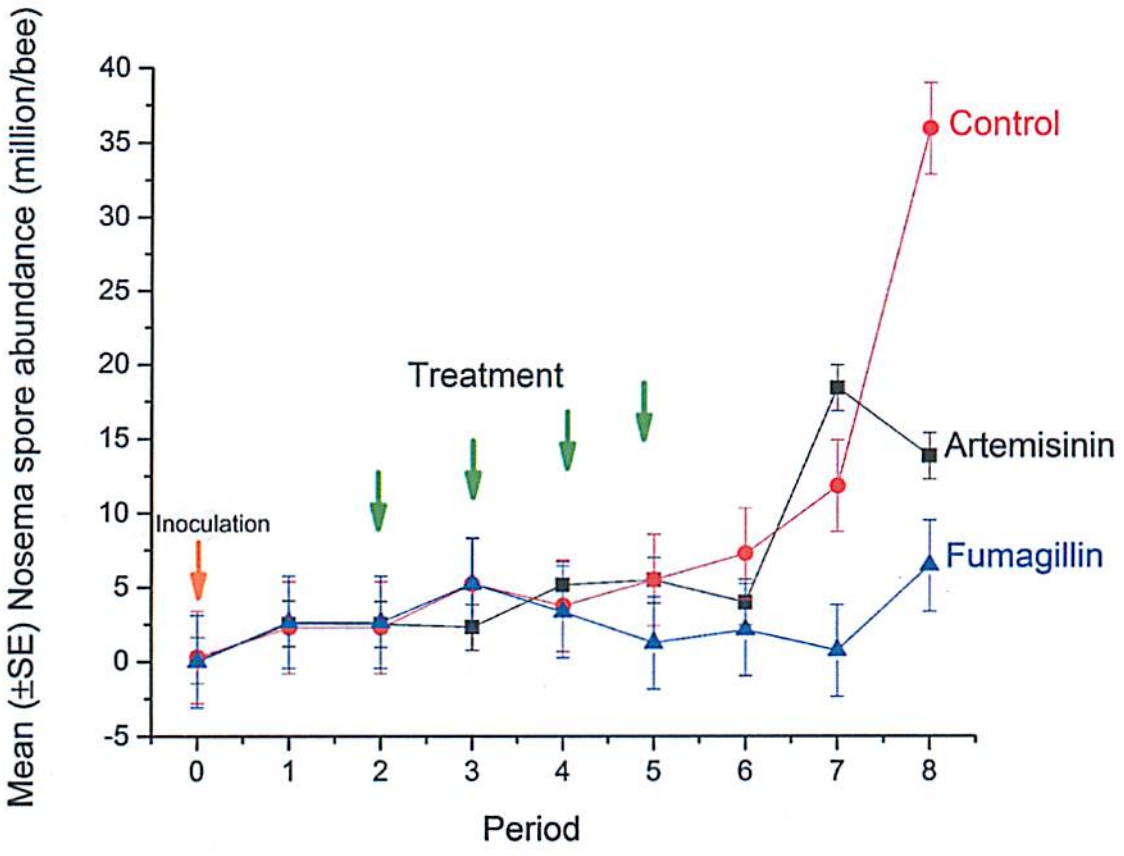

Figure A5.13. Mean (±SE) *Nosema* spore abundance (million per bee) in bees collected from nucs (2018). Arrows indicate time points of inoculation and treatments.

Appendix 6: Objective 3 Evaluation of factors affecting *Nosema* outbreaks and annual winter mortality

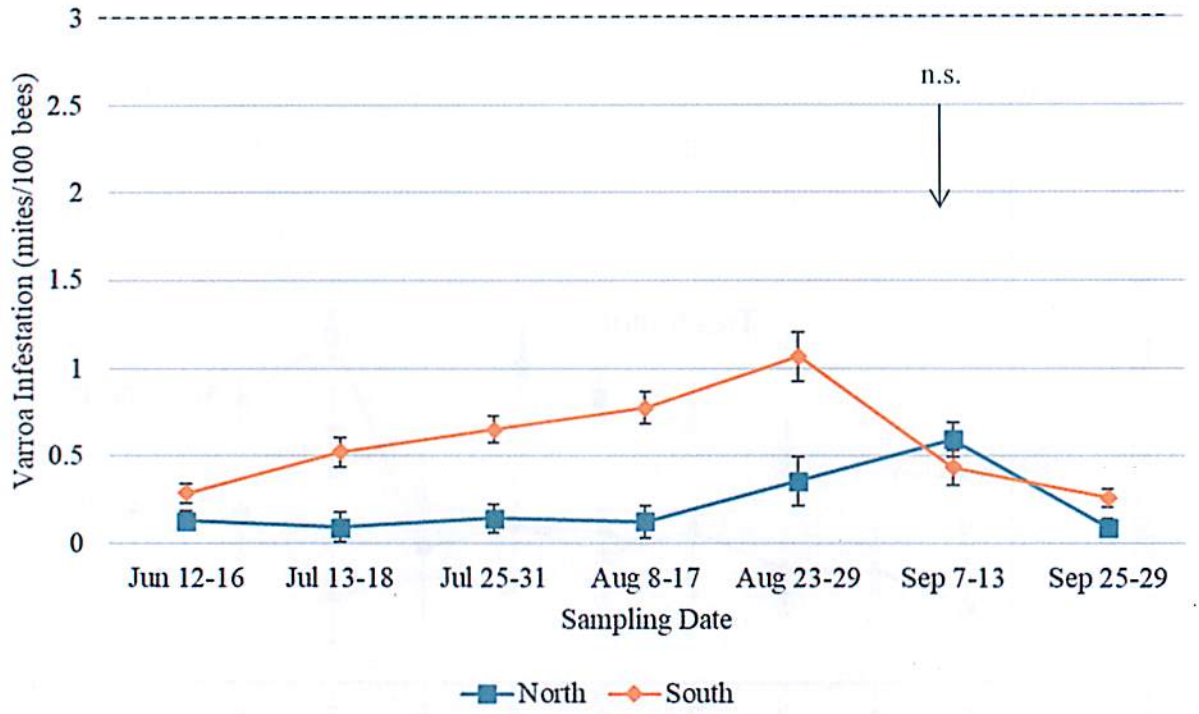

Figure A6.1. Average *Varroa* infestation over time by location. Arrow indicates when Apivar® was applied. The dashed line indicates the economic threshold. Significant differences were found at all sampling points except that indicated by n.s. (not significant).

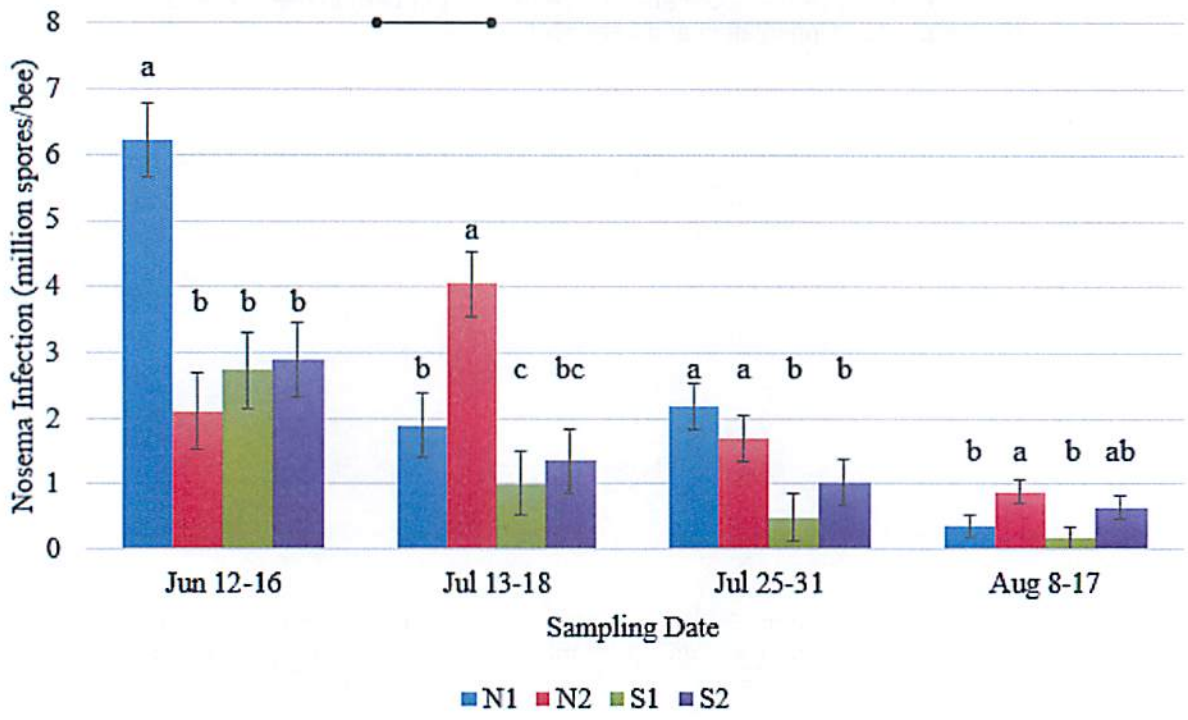

Figure A6.2. Mean *Nosema* abundance over time by apiary. Significant differences between apiaries are indicated by letters. Bar indicates when spring treatment was given.

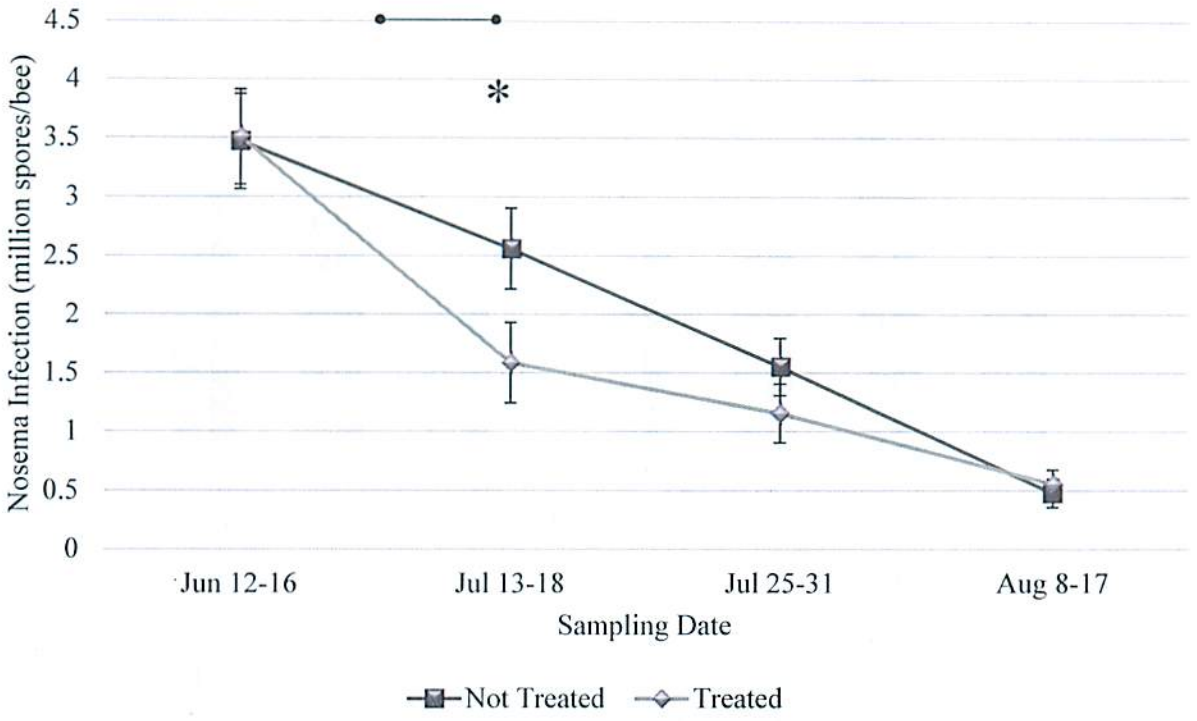

Figure A6.3. Mean *Nosema* abundance over time by colonies treated or not treated in the spring. Bar indicates when treatment was given. Significant differences indicated by an asterisk.

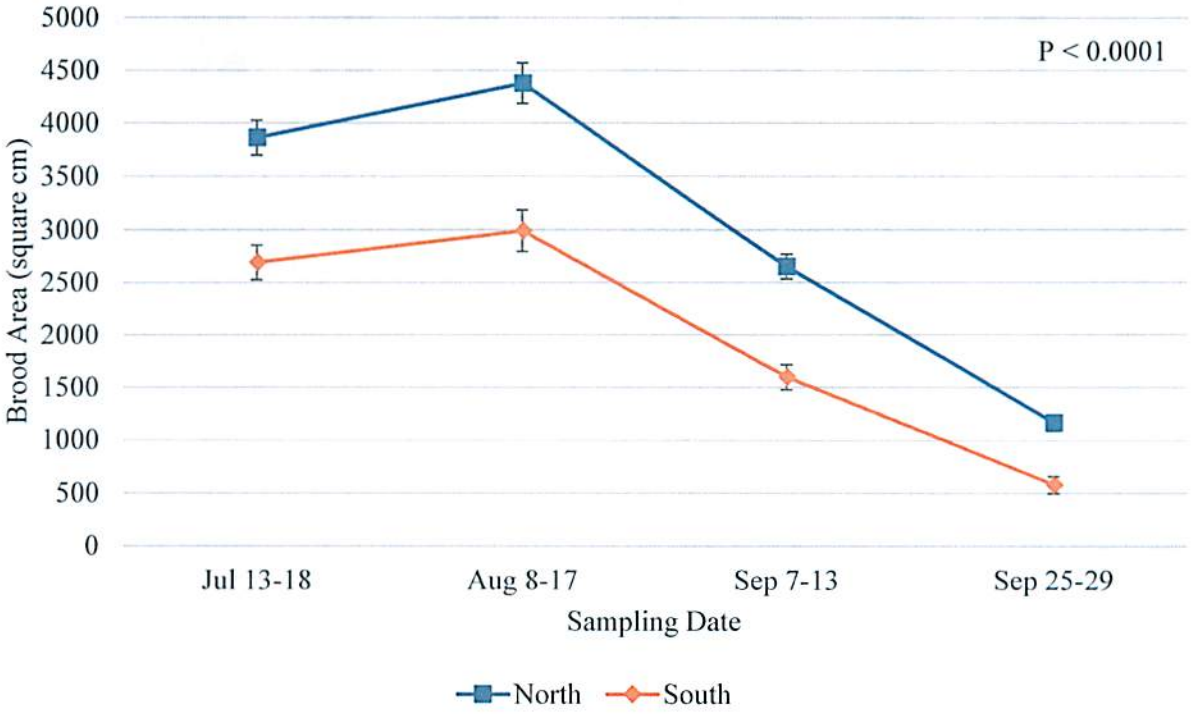

Figure A6.4. Brood area over time by location. For all dates, there were significant differences between locations.

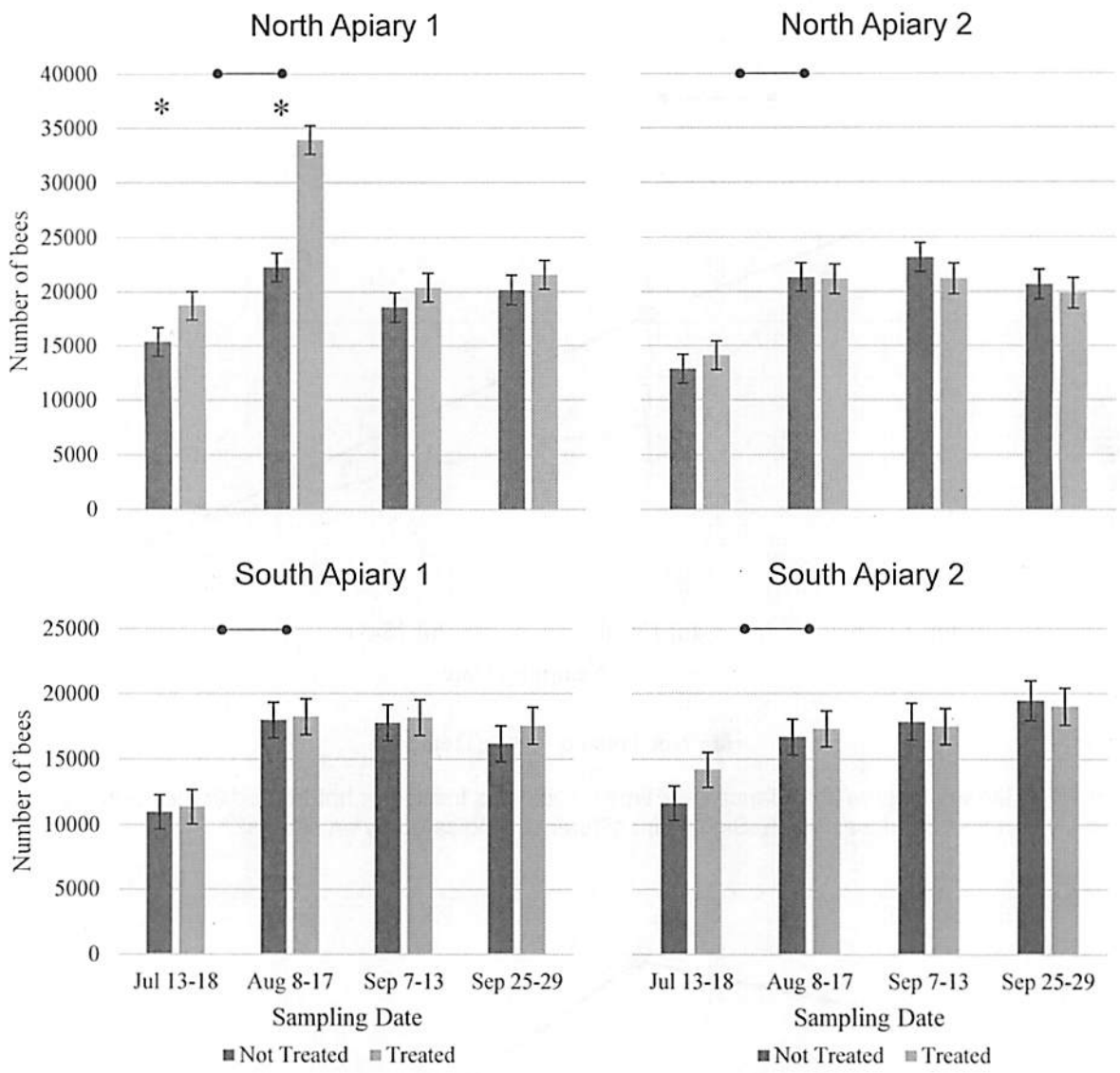

Figure A6.5. Bee population over time by colonies treated or not treated in the spring for each apiary. Bar indicates when treatment was given. Significant differences indicated by an asterisk.

Table A6.1. Number of live colonies before and after winter, winter mortality, and non-viable colonies for each apiary.

Apiary	Before Winter (Sept 25-29)	After Winter (Apr 24/26)	Winter Mortality (%)	Non-Viable Colonies
N1	39	31	20.5	2
N2	36	32	11.1	11
S1	35	23	34.3	14
S2	34	30	11.8	8

Table A6.2. Number of live colonies before and after winter, winter mortality, and non-viable colonies by location and wintering method.

Location/ Wintering	Before Winter (Sept 25-29)	After Winter (Apr 24/26)	Winter Mortality (%)	Non-Viable Colonies
North	75	63	16.0	13
South	69	53	23.2	22
Indoor	70	62	11.4	19
Outdoor	74	54	27.0	16

Table A6.3. Number of colonies that died over winter per treatment by location, wintering method, and overall.

Treatment	North	South	Outdoor	Indoor	Overall
Spring only	1	5	5	1	6
Fall only	3	1	3	1	4
Spring/Fall	4	4	4	4	8
Control	0	4	4	0	4

Table A6.4. Number of non-viable colonies per treatment by location, wintering method, and overall.

Treatment	North	South	Outdoor	Indoor	Overall
Spring only	3	3	3	3	6
Fall only	4	7	5	6	11
Spring/Fall	2	2	2	2	4
Control	2	3	0	5	5

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