Effect of Oxalic acid on *Varroa destructor* and Virus Replication in Honey bees

By

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A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

Department of Entomology

University of Manitoba

Winnipeg, Manitoba, Canada

Dedication

To my parents

my mother, whose strength held me together,

and my father, whose memory walks beside me every day.

To my elder brother and his wife,

whose love and guidance lit the way.

To my twin brother and my sweet nephew,

for lifting my heart when it felt heavy,

and reminding me to smile through it all.

This work is rooted in your love,

and I carry it forward for each of you.

Acknowledgments

This thesis would not have been possible without the support and encouragement of many individuals who contributed in various ways throughout this journey.

First and foremost, I am deeply grateful to my advisor, Dr. Rob Currie, for his unwavering support, patience, and guidance throughout this project. His expertise in honey bee research and his readiness to assist with challenges at every stage were invaluable. I would also like to express my sincere thanks to my committee members, Dr. Kyle Bobiwash and Dr. Byron Van Nest, for their time, thoughtful feedback, and continued support throughout the course of my graduate program.

I am thankful to Kathy Graham and Erika Hart for their assistance with administrative and office-related tasks. I am also indebted to the Bee Lab team for their help in the field and laboratory. I would like to acknowledge Zoe Rempel and Kira Peters, along with summer students Paul Grubiak and James Watson, for their assistance with data collection, and daily research activities. My appreciation also goes to our technicians, Dave Holder and Wonhyo Lee, whose support made this process more manageable.

I would like to express my sincere thanks to Provincial Apiculturist Derek Micholson and KRTP team members Matthew Polinsky and Amanda Turriff for their encouragement and support throughout this project.

I gratefully acknowledge the Department of Entomology Graduate Student Association (DEGSA) for fostering a supportive and vibrant graduate community. Special thanks to Sara Lavalley, Bridget White, Victoria Smelko, Cecil Montemayor Aizpurua, Danie Wood, Cassandra Madden, and Thilina Hettiarachchi for their friendship and shared moments of joy.

I am especially thankful to my friends Abhishek Dhawan, Neeraj Ghanghas, Kritika Sardool, and Naveen Kumar for their unwavering encouragement and companionship during some of the most challenging times of this journey. Their support meant the world to me.

This research was made possible through the generous financial support of the University of Manitoba Graduate Research Fellowship, the Canadian Agricultural Partnership, the Manitoba Beekeepers Association, and Bee Maid. I am sincerely grateful for their contributions.

Finally, heartfelt thanks to my family, friends, colleagues, and mentors who stood by me throughout this process. Your belief in me has been a constant source of strength.

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General Abstract

Honey bee colonies (*Apis mellifera*) continue to suffer high overwintering losses, with *Varroa destructor* mites and viruses like deformed wing virus (DWV) recognized as major contributing factors. Effective and timely fall treatments are necessary to reduce *Varroa* populations before winter without compromising colony health. This study examined the efficacy of oxalic acid (OA) treatments for controlling *Varroa* mites and their impacts on colony performance and virus dynamics. In the first set of experiments, colonies were treated once in early fall with 3.5% OA trickle, and monitored for changes in mite levels, virus titers, protein content, colony weight, and survival. Although daily mite mortality increased post-treatment, final fall mite abundance was not significantly reduced. However, surviving colonies treated with OA showed lower levels of DWV-A and sac brood virus (SBV) viruses in the spring. Cage experiments revealed that OA alone increased DWV-B levels in the absence of mites. Although it may have had some suppressing impact on block queen cell virus (BQCV), the buffer used for virus inoculation also showed independent virus-suppressing effects.

In the second set of field experiments, we evaluated the frequency, timing and interval between OA vapor applications in late fall and colony performance under different wintering environments. Colonies received zero, one, or four treatments at 2-5 day variable intervals in November in one experiment, and were treated four times either at 5-day or 12-day intervals spanning from September to November or concentrated at the end of the treatment window. Colonies were wintered either indoors or outdoors, and assessed for mite levels, cluster size, colony weight, survival, total protein, and viral load. Colonies treated at 5-day intervals showed better survival then controls for the colonies with low initial mites. While both one and four applications caused mite mortality, only four applications significantly reduced *Varroa* populations by spring as measured by alcohol wash. Treatments did not directly affect

protein or virus levels, but colonies with low initial mite loads had reduced DWV-B levels when treated. Indoor wintered colonies had consistently lower DWV levels than outdoor-wintered colonies. Overall, repeated OA vapor treatments in late fall helped reduce *Varroa* loads and supported colony survival, but their effectiveness depended heavily on initial infestation levels and treatment timing. These findings highlight both the potential and limitations of OA as a late-season *Varroa* control strategy.

General Introduction

Varroa destructor is a major threat to honey bee colonies . In this thesis, I tested how oxalic acid (OA) affects *Varroa* mites and viruses in honey bees. *Varroa destructor* is an ectoparasitic mite that feeds on the haemolymph and fat bodies of honey bees. We treated honey bee colonies with OA using both vaporization and trickling methods to assess its effects on *Varroa* mites. Moreover, we conducted cage experiments to investigate the effect of OA on viruses.

In the first chapter, I provided a brief overview of honey bees and how *Varroa* mites and viruses affect their survival. I also outlined the objectives and hypotheses of the study. In the second chapter, which is to be submitted for publication in a scientific journal, I report on a series of field and lab experiments exploring the effects of OA trickle formulations on colony health and viruses. In an early-fall field experiment, we treated 20 colonies with 3.5% trickled OA, while 20 colonies served as controls. We monitored *Varroa* mite levels using sticky boards beneath the hive to monitor mite drop and by collecting adult honey bees to assess varroa abundance by alcohol wash. I also collected bee samples to analyze virus levels. Additionally, other colony parameters associated with colony health such as colony weight, cluster size, and protein levels were recorded. We observed that the daily mite mortality rate increased two weeks after OA application. Furthermore, OA treatment reduced sac brood virus (SBV) and deformed wing virus strain -A (DWV-A) virus levels in the spring. Colony survival was most affected by *Varroa* mite levels and DWV-A. For the lab experiment, we used Australian honey bees to assess the effects of OA under a *Varroa*-free environment. This in vitro experiment was conducted using worker bees in plexiglass cages. Honey bees were treated by pipetting OA onto individual bees in a dose designed to mimic the trickle treatment. Bees were successfully orally inoculated with DWV-A and DWV-B viruses. The buffer (0.1 M ascorbic acid and 0.5 M potassium phosphate) in the inoculum independently reduced

the levels of SBV and BQCV. We observed that OA reduced black queen cell virus (BQCV) levels but increased DWV-B levels.

In the third chapter, which also is to be submitted for publication in a refereed journal, I conducted two field experiments using doses of 1 g of OA vapor applied in late fall under different wintering environments. In the first experiment, conducted in 2020, we applied vaporized OA once to 20 colonies, four times at variable intervals (2–5 days) to another 20 colonies, and 20 colonies served as controls. In the second experiment, conducted in 2021, we treated 20 colonies at 5-day intervals, 20 colonies at 12-day intervals, and included 20 control colonies. During winter for both experiments, half of the colonies from each group were placed in indoor, and the other half in outdoor, wintering environments. *Varroa* levels were monitored using sticky boards and by collecting mite samples. Virus levels, protein content, cluster size, and colony weight were also measured. Mite mortality increased in colonies treated with either a single dose or four doses in the fall. Colonies with low mite infestation showed higher survival rates when treated at 5-day intervals than when treatments were spread out throughout fall at 12 day intervals. DWV levels were lower in colonies with low mite infestations and were also lower in indoor-wintered colonies compared to those wintered outdoors.

In the fourth chapter, I discussed the overall interpretation of the thesis results and provided possible explanations.

Chapter 1: Literature Review

Introduction to Industry

European honey bees (*Apis mellifera* L.) are distributed worldwide and found on most continents except for Antarctica (Moritz et al., 2005). The global expansion of honey bees happened due to human assisted movement of colonies (Boncristiani et al., 2020). While bees have been exploited by humans in various cultures, modern beekeeping became possible due to the invention of Langstroth hives with movable frames in 1851 that allowed colonies to be inspected and manipulated more effectively (Chauhan et al., 2021).

Honey bees are ecologically and commercially important. They are helpful for pollination of native flora especially in their ancestral areas as well as crops and produce many commercially essential products like honey, wax, royal jelly, propolis, and bee venom (Bobiş et al., 2010; Sahinler & Kaftanoglu, 2005; Wehbe et al., 2019). Honey bees are commercially valuable as they are well-suited to be used as pollinators of many monoculture crops (Calderone, 2012). One-third of the human diet relies on pollination by honey bees (Delaplane & Mayer, 2000). Honey bees contribute to human nutrition by providing components of a balanced diet by pollinating crops such as berries, vegetables, orchard fruits, and hybrid canola. Although, there are also many staple crops (rice, wheat, potato, and corn) that are wind-pollinated and independent of honey bee pollination (Richards, 2001). According to report by Agriculture and Agri-Food Canada report, (AAFC (2023), the estimated value of honey bee pollination is $7 billion annually in Canada alone. Total honey production in Canada in 2023 was 91,807,000 pounds of which 18.8% was produced in Manitoba. Canada exports $46 million worth of honey, 37% of which was exported from Manitoba (AAFC, 2023).

Unfortunately, honey bees face annual colony losses due to pests, pathogens, pesticides and weather conditions, with rates significantly higher than those observed in the industry decades ago. For example, according to the Canadian Association of Apiculturists, national levels of colony loss after the winter of 2022 was over 45%, with provincial losses varying between approximately 15% and 57% (CAPA, 2022). In 2022, the loss of colonies nearly doubled the reported average annual loss from 2007 to 2021 (25.8%) and was substantially higher than historic numbers for expected colony losses that averaged 5 to 15 percent (Furgala et al., 1992). Although multiple stressors contribute to loss, ineffective varroa control and issues with associated virus infections are thought to be the leading causes of colony loss (Insolia et al., 2022; Flores et al., 2021).

Apis mellifera

Apis mellifera is a species of honey bee that is of European origin, and although not native to North America, it can withstand North American winters in northern regions with some assistance. In many regions of Canada, honey bees are protected from low temperatures either by placing them outdoors and wrapping them with insulating material or by placing them indoors in a wintering building maintained at 2ºC - 4ºC (Bahreini & Currie, 2015a; Gruszka, 1998). Honey bee colonies have three castes, which are the queen (fertile female), workers (sterile female), and drones (male). The age structure of workers in honey bees varies seasonally; they are long-lived in winters (>8 months), whereas their life span is shorter in summers (~4 weeks). Worker bees that emerge in fall and survive through the coldest months are known as winter bees (Mattila et al., 2001). Honey bees go through behavioral and physiological changes to become winter bees and then back to summer bees.

Behavioral changes in winter bees vs summer bees

During late spring, summer, and early fall, worker bees are short-lived; their life span is around 30 days (Döke et al., 2015). Seasonal resource availability, colony size, brood demands, and pathogen or parasite pressures influence this lifespan, but the timing of the shift from in-hive duties to foraging is a particularly strong determinant (Rueppell et al., 2007, 2008). In summer, they have a well established age-based division of labor that begins after the brood emerges from their cells as adults (Huang & Robinson, 1996). The workers first clean cells and seal the brood for the first few days (Seeley, 1982). Between 3-10 days of age, the workers act as nurse bees and start taking care of the brood and queen (Hrassnigg & Crailsheim, 1998b). When 11-20 days old, the workers store and process food, maintain the nest, construct a comb, and later act as guard bees to protect the hive from other predators and pests and defend the colony from robber bees from other colonies and other threats (Breed et al., 1990; Winston, 1987). At 19-21 days of age, they perform riskier behaviours and go outside to collect pollen, nectar, propolis, and water (Robinson, 1992). The collection of pollen and nectar is essential for colony growth and honey production; the colony stores large amounts of honey to be consumed as the only carbohydrate source for colony survival in winter (Döke et al., 2015). In North temperate climates, the foraging period decreases as the photoperiod shortens in late summer and available forage diminishes. Also, there is a reduction in foraging activity that occurs due to cooler environmental temperatures (Döke et al., 2015). At that time, worker bees start conserving their resources for winter by reducing brood rearing (Mattila & Otis, 2007). Workers also begin to store lipids and proteins in their bodies to “prepare” themselves for winter (Amdam & Omholt, 2002). During late fall the population of short-lived summer worker bees are gradually replaced by long-living winter bees (Fluri et al., 1982). This conversion to winter bees helps colonies survive during less favorable conditions like low food availability and temperatures (Ricigliano et al., 2018). In northern climates, like in Manitoba, colonies going into winter during the months of

September and October and coming out of winter in April and May have both winter and summer honey bees in the colony (Kunc et al., 2019). As the seasonal changes vary with geographical location, the timing of occurrence of summer and winter bees would also need to vary so the use of a single fixed type of cue like photoperiod to initiate this is unlikely (Fukuda & Sekiguchi, 1966). In northern temperate climates as brood production slows down in autumn and then halts and older summer bees die off, the size of the colony decreases to ~15,000-20,000 bees from a summer peak population of up to 70,000 bees (Van Nerum & Buelens, 1997).Worker bees also stop rearing drones and forcibly remove adult drone bees from the hive in fall (Mattila et al., 2001).

In Manitoba, the minimum outdoor temperatures can go lower than -38°C, and honey bee workers individually cannot cope with these extreme temperatures. However, honey bees as a colony, can act as a super-organism and survive such unfavorable climatic conditions by forming clusters of workers and maintain temperatures at ~30-34 °C even under extremely cold ambient conditions (Jones et al., 2004). The bees begin to form thermoregulated clusters in the hive, when the ambient temperature falls below 10 °C (Mattila & Otis, 2007). Under extreme cold, the outer edge temperature of the cluster is maintained at 6-12°C by the vibration of the flight muscles of clustered bees to generate heat. This helps to keep the temperature suitable for the survival of bees on the outer edge (Stabentheiner et al., 2003). Honey bees use the supplies of honey that were collected and stored during summer; this is the only source of energy available for consumption in winter without the provision of supplemental feeding by beekeepers (Amdam et al., 2005; Döke et al., 2015). During these extreme cold climatic conditions, ”winter” honey bees typically remain in the hive for the duration of winter to allow survival of the colony (Seehuus et al., 2007). Brood production can occur during winter (Kozak, 2008) but resumes at a high level when the temperature is warmer and forage is available, restarting the division of labor and the annual colony cycle. When the brood rearing initiates, in winter, the surrounding brood area temperature

is maintained at up to ~33°C (Moeller, 1977). A large colony cluster size (9-10 frames) is essential for the colony to be able to survive until spring.

Physiological change in winter bees vs summer bees

Honey bees undergo physiological changes during different seasons to allow for the longer life span described above. The fat body is the main regulatory organ for metabolism and storing nutrients in honey bees; this tissue is in the abdomen and, to a small degree, in the head of honey bees (Münch et al., 2015; Seehuus et al., 2007). Vitellogenin is the main nutrient storage lipo-protein in honey bees; it is synthesized from fat bodies (G. V. Amdam et al., 2012). Vitellogenin varies seasonally and in spring and summer, production is higher for ten days after the emergence of the honey bee (R. F. Chapman, 1998). During this period, as honey bees consume pollen, the hypopharyngeal gland grows (Corby-Harris et al., 2022). Hypopharyngeal glands are in the heads of honey bees and used to produce brood food. The size of hypopharyngeal glands is larger in nurse bees (2-11 day age) in comparison to older worker bees that perform other hive duties, foraging and guarding behaviors (Corby-Harris et al., 2022). Royal jelly (a component of brood food for both workers and queens) is also synthesized from vitellogenin in the hypopharyngeal glands of nurse honey bees (G. V. Amdam et al., 2003). Nurse bees then provision very young worker larvae with protein rich worker jelly (Snodgrass, 1956). When nurse bees feed other members of the colony, the vitellogenin utilization exceeds production by the fat body, leading to diminishing the vitellogenin levels in the hemolymph (Smedal et al., 2009). As bees age, however, the hypopharyngeal glands shrink (Hrassnigg & Crailsheim, 1998), and the fat bodies diminish (Toth & Robinson, 2005). As a result, the hemolymph of nurse bees has higher levels of vitellogenin and total protein than the hemolymph of foraging bees. When the honey bee transitions from nursing to a foraging state, the levels of juvenile hormone also rise in the hemolymph. The vitellogenin and juvenile hormone are in a negative feedback loop, which means when levels of juvenile hormone increase, the levels of

vitellogenin are suppressed, and when the levels of vitellogenin decrease, the levels of juvenile hormone increase (Döke et al., 2015). Honey bees use Juvenile hormone III which is synthesized within the corpora allata, and it is the only form of juvenile hormone found in honey bees (Robinson, 1992). Age related task division in honey bees and associated with glandular changes described above are linked with the levels of juvenile hormones (Knoll et al., 2020). The critical cause for physiological change in honey bees resulting in higher vitellogenin levels when transitioning to winter bees is thought to be linked to a decrease in brood rearing (Mattila & Otis, 2007). Due to the decrease in brood rearing, the worker bees do not need to provide feed to the brood, which decreases the nutritional demands on worker bees. The bees thus could end up having more nutritional stores in their bodies. Because of more nutritional stores, vitellogenin levels and total protein levels can increase (Fluri et al., 1982) and without juvenile hormone suppression, the production of vitellogenin continues, allowing bees to keep storing nutrients and leading to high longevity (Fluri et al., 1977; Smedal et al., 2009).

Brood rearing is initiated on a large scale at the end of winter to create a new generation of honey bees that replace winter bees (Mattila et al., 2001). The impact of brood rearing in winter on the physiology of winter bees is not well understood. The life expectancy of winter bees can vary with vitellogenin level (Dainat et al., 2012a). Knowledge of the timing of winter bee formation is important in deciding treatment timing with acaricides to control varroa. For example, Dooremalen et al. (2012) showed that the winter survival of honey bees could be increased when colonies were treated for mites earlier in a season, which reduced the varroa mite infestation in time to presumably enhance the production of long lived winter bees. Timing and type of mite applications also affect the age structure of colonies going into winter in the Canadian prairies (Parsons, 2017).

Varroa destructor

Varroa destructor (Anderson and Trueman), earlier known as *Varroa jacobsoni* Oud., was a parasite on its Asian honey bee host *Apis cerana* Fabricus. Varroa mites shifted to a new host, the European honey bee in the 1940s when these bees were brought to Asia for apiculture (Warner et al., 2023). On the North American continent, varroa infestation was first noticed in Florida in 1987, from where it spread across other states in the US (de Guzman & Rinderer, 1999). It was first detected in Canada in 1989 (Currie et al., 2010). Varroa mites are obligate ectoparasites that cause damage by consuming the hemolymph and fat bodies of the pupal and adult stage honey bees (Ramsey, Ochoa, Bauchan, Gulbronson, Mowery, Cohen, Lim, Joklik, Cicero, Ellis, Hawthorne, & vanEngelsdorp, 2019; Rosenkranz et al., 2010). The lifecycle of the mite has two phases- the reproductive phase and the phoretic (female adult stage on adult bees) phase (Rosenkranz et al., 2010). During the phoretic stage, varroa feeds primarily on nurse honey bees while they are tending the larvae but can move to other honey bees involved in grooming or foraging (Peck et al., 2016). Varroa mites have a strong preference for drone brood cells in comparison to worker brood cells for reproduction (Martin, 1995). The infestation rate is 8-10 times higher in drone brood. Varroa preferentially select drone brood over that of workers as the drone pupae take a longer time to develop, enabling more of the varroa mites that are produced to reach maturity (Calderone & Lin, 2001).

During summers, the phoretic phase lasts 4.5-11 days, while in winter, the phoretic phase lasts up to six months (Ellis & Nalen, 2022). The mated female varroa mite (foundress) enters the brood cell before it is capped (DeGrandi-Hoffman et al., 2017). About 60 hours after the brood cell gets capped, an egg is laid by the foundress mite, which is haploid and develops into a male mite. Later, the foundress mite lays diploid female eggs every 30 hours, and these eggs hatch and grow through several molts to the adult stage (Traynor et al., 2020). During the reproductive phase, the varroa mite and its offspring feed on

the hemolymph and fat body of pupae, consuming essential nutrients and cause some damage to the developing honey bees (Le Conte et al., 2010). Development of immature nymphs to the adult stage of varroa takes 5-8 days, then the adult male mite mates with any mature sister mites (those that can have fertile offspring). The foundress mite and some daughter mites exit the cell with the emerging honey bee (Roth et al., 2020). Emerging honey bees may be weak due to previous feeding from varroa mites, as both the foundress mite and her offspring feed on pupae and the emerging bee. After the workers or drones emerge, any immature female mites and male mites left in the cell die. The phoretic mated female mites and original foundress can transfer from one worker bee to another and feed until a new host cell is found. Varroa generally attach themselves to nurse bees, which tend to brood and increase their chances of finding a new host cell (Xie et al., 2016).

Viruses

Varroa mites are vectors for honey bee viruses like deformed wing virus (DWV), Israeli acute paralysis virus (IAPV), Sac brood virus (SBV), Kashmir bee virus (KBV), acute bee paralysis virus (ABPV) (Boecking & Genersch, 2008). Varroa mites can transfer viruses to a new host after 36 hours of being infected by the virus (Yañez et al., 2020). Varroa populations and viral loads can be positiverly correlated for some of the virues within honey bee colonies (Tibatá et al., 2021).

Most of the viruses that infect honey bees are single stranded positive sense RNA viruses like deformed wing virus (DWV), black queen cell virus (BQCV), Israeli acute paralysis virus (IAPV), sac brood virus (SBV), acute bee paralysis virus (ABPV), and Kashmir bee virus (KBV) (Brutscher et al., 2015). Viruses alone can impact honey bee foraging, hive defense, flying capacity, immunity, and the life span of individual workers, leading to a decline in the colony's health and colony collapse (Ullah et al., 2021). These viruses can affect all life stages of honey bees, such as eggs, pupae, and larvae, and adults as

well as all casts, (queens, drones, and workers) (Y. Chen et al., 2004). Some virus infections show visible symptoms (overt), and some will exist in the bees without outward symptoms (these are considered to be covert). The viruses can be transmitted horizontally or vertically (Boecking & Genersch, 2008). In horizontal transmission, they are transmitted from one bee to another in the same generation through multiple routes (Chagas et al., 2019). Viruses can be transmitted horizontally by varroa, oral foodborne routes, fecal-oral routes, or by direct contact (Bowen-Walker et al., 1999). For vertical transmission, the viruses are transmitted from the queen and drone to their offspring, either trans ovum (virus on the egg surface) or transovarial (virus within the egg), or venereally (through sperm) (Amiri et al., 2016; Y. Chen et al., 2006). Virus infection can have lethal or sublethal effects as varroa mites that feed on the pupae or adults weaken their immune systems and make bees susceptible to other virus infections (de Miranda et al., 2013).

This thesis focuses on the following five economically important viruses that are common in Canada – DWV-A, DWV-B, BQCV, IAPV, and SBV (Desai et al., 2016). Colonies infested with varroa are often infected by one or more variants of the deformed wing virus DWV. Varroa mites act as mechanical vectors and can transmit DWV while feeding on honey bees (Shen et al., 2005). DWV can be transmitted from varroa mites to pupae, causing malformation and death of pupae (de Miranda & Genersch, 2010). The symptoms of a shortened abdomen and deformed wings are only found if the transmission happens through the pupae (Tentcheva et al., 2006). However, varroa mites also act as biological vectors, as some forms of DWV can replicate inside varroa, which is considered the cause of overt DWV infection in honey bees (Gisder et al., 2009). If the varroa levels are controlled chemically, existing DWV in colonies can be transmitted by consuming infected pollen and through trophallaxis to larvae (Locke et al., 2017). Deformed wing virus is a complex virus with different variants and strains (DWV-A, DWV-B, and DWV-C) that vary in virulence (Kevill et al., 2019). In previous studies, DWV-B

was demonstrated as more harmful than DWV-A (Natsopoulou et al., 2017) but now Kevill et al. (2019) showed DWV-A is more virulent. DWV-B was previously called *Varroa destructor* virus 1 (VDV-1), as it is strongly associated with varroa and replicates in the mite (Ongus et al., 2004). If the pupae are infected with either DWV strain, they show similar mortality and levels of wing abnormalities for both DWV-B and DWV-A strains (Norton et al., 2020). These strains are present in various parts of the honey bee with slight differences in location; DWV-A is usually more commonly found in the head and legs, while DWV-B is more commonly found in the head and abdomen (Penn et al., 2022). DWV shows a significant correlation with winter loss of honey bee colonies (Berthoud et al. 2010, Desai & Currie 2016, Claing et al. 2024).

Black queen cell virus (BQCV) is an RNA virus from the family *Dicistroviridae* (Spurny et al., 2017) . It was first isolated from queen cells with dead prepupae and larvae that were black in color but is also found in adult worker bees (Leat et al., 2000). According to (NBDC, 2017), BQCV was tested in seven provinces of Canada and was very common with 90-100% prevalence. The virus is transmitted to larvae while getting fed by glandular secretions of nurse bees (Bailey & Ball, 1991). Adults do not show external symptoms due to BQCV infection (Tentcheva et al., 2004). No conclusive result shows varroa is a vector of BQCV (Mondet et al., 2014). Black queen cell virus is detected in all life stages of all the honey bee casts (worker, queen, and drone) and may interact with other pathogens such as nosema, but it can be lethal in the queen (Cornman et al., 2012; Murray et al., 2019).

Israeli acute paralysis virus (IAPV) has been directly linked to colony losses (Desai & Currie, 2016a; Di Prisco et al., 2011). It was first detected in Israel (Maori et al., 2007). The infected bees show trembling and shivering wings; honey bees are paralyzed and found dead at the entrance of the colony (Cox-Foster et al., 2007). IAPV belongs to the family *Dicistroviridae,* and viruses like kashmir bee virus (KBV) and acute bee paralysis virus (ABPV) belong to the same family and are not clearly represented as

separate from IAPV, but they are considered complex or closely genetically related to each other (de Miranda et al., 2010).

Sac brood virus (SBV) was first detected in the US in 1913, (White, 1913) in immature bees. However, Sacbrood virus is now known to infect both adult bees and brood; the two day old larval stage is more vulnerable to getting infected by SBV (Bailey & Ball, 1991). The infected larvae form fluid filled sacs and fail to pupate, in which SBV multiplies and produces millions of SBV particles (Hitchcock, 1966). Nurse bees get contaminated by SBV when they remove the SBV-infected dead larvae, and the SBV is transferred to healthy larvae while feeding them. In adult honey bees, SBV is symptomless but decreases the life expectancy (Bailey & Fernando, 1972).

Varroa Control

Varroa is the biggest threat to the honey bee industry. If the varroa is not controlled in an infected colony, the colony can collapse within six months to 3 years (Rosenkranz et al., 2010). Therefore, varroa mite levels should be monitored; if the spring or fall economic thresholds are exceeded, varroa control methods should be used to prevent productivity losses or colony death (Currie 2008). For the Canadian prairies in spring, the economic threshold level is >1%, for early fall >3%, and for late fall >10% (Currie, 2008) assuming no other stressors are present. When levels are above this varroa control is essential for honey bee colony winter survival. Various cultural, mechanical, and chemical methods have been used to control varroa mites (Jack & Ellis, 2021). Cultural methods include breeding mite-resistant bees. Natural honey bee defense against varroa is often through forms of social immunity and bees can be selected for increased propolis collection (Simone-Finstrom et al., 2017), increased hygienic behavior (Harbo & Harris, 2009) or increased grooming (Micholson & Currie, 2024; Morfin, Harpur, et al., 2023). Another cultural method is brood interruption; the queen bee is caged to stop it from laying eggs and create an

artificial brood break, which interrupts the growth of varroa mites (Wagnitz & Ellis, 2010). For mechanical control, drone brood removal can be used (Sammataro et al., 2000). It is based on the varroa mite's preference for infesting developing drones, so in practice, a frame containing drone brood is placed in the hive to attract and trap varroa mites before cell capping; the frame is removed from the hive after cell capping and before the emergence of drones then killed. Another mechanical method is using screened bottom boards instead of solid ones (Delaplane et al., 2005). The screened bottom board is placed under the hive; sticky paper can also be placed under the screen with a sticky side facing upward so the mites cannot crawl back up through the screen. Honey bees entering the hive do not come in contact with the fallen mites, preventing the mites from reinfesting the hive. When natural mite fall occurs, the mites get stuck on the bottom board and cannot re infest the hive.

Traditionally, varroa mites have been controlled by synthetic chemicals with active ingredients such as tau-fluvalinate, flumethrin, amitraz, and coumaphos that are used as registered pesticides for other insects or mites (Rosenkranz et al., 2010). However, varroa has developed resistance against all these classes of synthetic chemicals (Elzen et al., 1999; Pettis, 2004; Rinkevich, 2020). Since these chemicals are insecticides, they do have some level of toxic impact or sublethal effects on honey bees and can cause some potential for residue accumulation in honey and wax (Gregorc & Planinc, 2001). That is why there is increased interest in using "organic" chemicals like oxalic acid (OA), formic acid, and lactic acid as well as other essential oils to control mites. Although the acids are very effective and fairly safe to use; if overused, they too may lead to the mortality of bee brood, supersedure, and some bee mortality especially when they need to be applied under variable environmental conditions (Underwood et al., 2019). For this thesis, I will focus on the use of OA. OA is used worldwide and is one of the most widely applied organic acids against varroa (Van der Steen & Vejsnæs, 2021).

In Europe, OA has been used since 1980; based on research done in Eastern Europe and Asia, delivery methods involve spraying, trickling, and sublimation ("vaporizing") of the acid (Nanetti et al., 2003). As the spraying technique was time consuming, a more practical and time efficient method was developed, using trickling methods (Nanetti and Strada 1997). Over time, it became popular among Western Europe beekeepers due to its high efficacy in broodless colonies (Imdorf, Bogdanov, et al., 1995). NImdorf, Bogdanov, et al., 1995).When applied at the colony level in honey bees the OA does not penetrate cell capping, so it is best to apply during the broodless period (Rademacher & Harz, 2006). In Canada, it is currently registered to be applied in three different ways, although registration of other delivery methods is being pursued. Producers can currently apply it by spraying OA in an aqueous solution, trickling OA in a sugar solution, and sublimation using a variety of applicators that heat the product to sublimate (vaporize) it. The recommended dose for an aqueous solution of OA is 35 g OA in 1 L of 50% sugar syrup (Micholson, 2024)**.** For the spraying method, a pressurized hand sprayer is used to spray OA solution, each frame is removed one at a time and sprayed: this is done for every frame in the hive (Al Toufailia et al., 2015) and quite labour intensive. In the Netherlands, Denmark, and Sweden, the trickle method is common, and producers apply a 3.2% w/v OA solution in 50% sugar syrup during winter to ensure low mites in spring (Van der Steen & Vejsnæs, 2021). In the trickling technique, 5 ml of 3.2 to 4.2% OA in 60 % sugar syrup is trickled in the space between frames (on the top bars) by using a syringe or commercial applicator. Honey bee hives typically have ten parallel frames. The frames are separated by three-eighths of an inch; this space is called bee space. During winters, the capped brood is absent or minimal. If the brood is present, it is recommended to remove the brood to improve the efficacy of the OA treatment. Practical experiences show that OA can be applied at lower temperatures of -5 degrees Celsius (Van der Steen & Vejsnæs, 2021). Oxalic sublimation is another popular way of treating varroa mites. In this method, the hive is closed, and any gaps are sealed with rags. Then, a vaporizer (*e.g.*Varroax® / ProVap®) is used to heat the OA, which converts the OA into a "vapor". In Canada, the

registered dose for the OA vapor method is 1g for a single brood chamber hive and 2g for a double brood chamber hive (Micholson, 2024).

Virus Control

As described above, virus infections are detrimental to honey bee colonies. Honey bees are social insects, so the pathogen and parasite control can occur not only through individual immune responses but also through social immunity. Social immunity is a collective set of actions that reduces disease transmission within the group (Cremer et al., 2007). To suppress viruses through social immunity, honey bees can be encouraged to collect propolis, which has antimicrobial and antiviral properties (Borba et al., 2015). Propolis is a mixture of plant resin and wax; plants produce resins to seal wounds and protect them from pathogens and pests (Simone-Finstrom & Spivak, 2010). Other forms of social immunity that either remove infected brood and bees or remove varroa can also help to suppress and control viruses (Simone-Finstrom et al., 2017).

In addition to these innate defenses, various human applied interventions aim to control honey bee viruses directly or indirectly. A molecular method could be used to control virus levels, Double stranded RNA (dsRNA) is used to silence the targeted genes using RNAi (RNA interference), which is a natural defense mechanism (Brutscher & Flenniken, 2015). Honey bees are orally delivered with exogenous double stranded RNA (dsRNA), which supplements endogenous RNA interference machinery to eliminate DWV (Desai et al., 2012), SBV (Zhang et al., 2016), and IAPV (Maori et al., 2009). Also, many chemicals used to control varroa have an impact on viruses since many viruses are vectored by varroa. Studies show that using short term thymol (Phytochemical) for varroa treatment also reduces the levels of DWV (Palmer-Young et al., 2017). However, some or all of the control of viruses may occur indirectly by controlling varroa mite levels which vector the viruses or suppress the bees immune system

allowing viruses to multiply. The impact of OA on controlling viruses directly or indirectly (by controlling mites) is not well understood.

Summary

Honey bees are important for pollination and honey production, playing a vital role in the economy and ecology of Canadian agriculture. However, beekeepers are repeatedly experiencing high annual losses of colonies, especially during overwintering. A major factor in this decline is due to the *Varroa destructor* mite, which feeds on honey bees and acts as a vector for harmful viruses like deformed wing virus (DWV). Varroa causes physical damage to honey bees and make them susceptible to other pathogens. Managing varroa is challenging because the mites have developed resistance to many synthetic acaricides. Organic treatments like OA are good alternatives, but OA has limitations since it cannot expose mites when present in capped brood cells. It works best during broodless periods in late fall and winter. More research is needed to determine the optimal timing and application of OA for varroa control and if OA can suppress viruses in colonies. Since varroa mites are a major cause of honey bee colony losses, improving control methods is crucial for the health of honey bees and the sustainability of agriculture.

Research gaps

While there has been extensive research on using OA to control varroa mites, the exact mechanism of how OA affects the mites is still unclear (J. D. Evans & Cook, 2018; Toomemaa, 2019). Parsons (2017) found that early fall applications of OA did not kill the mites but improved honey bee survival. This raises the question of how OA benefits honey bees even in cases when it has limited effectiveness against varroa mites in early fall. One theory is that OA may interfere with virus inoculation

or replication, this could help to make winter bees healthier going into winter. Although the interactions between honey bee viruses and synthetic acaricides have been studied, there is little research on how honey bee viruses interact with OA (Bubnič et al., 2024; K. C. Evans et al., 2022). Past studies have tested different doses and application methods of OA, and some have examined the effectiveness of synthetic acaricides in various wintering environments (Al Toufailia et al., 2015; Desai & Currie, 2016a; Gregorc & Planinc, 2001; Nanetti et al., 2003). However, more research is needed to study how OA applications at different intervals in various wintering conditions effects varroa mortality and honey bee health.

Objective

In this thesis I will investigate the mechanisms through which honey bees benefit from OA trickle treatment in early fall, despite its low efficacy against varroa mites. This research will assess virus replication both naturally within the colony and in controlled cage trials without varroa mite interference, to understand how OA influences virus transmission and enhances overall bee health.

I will also evaluate the impact of different application intervals of OA treatment using the sublimation method on its efficacy against varroa mites and viruses, and to assess its effects on the health of honey bee colonies managed under both outdoor and indoor wintering conditions.

Hypotheses

OA disrupts viral replication in honey bees. If viral replication is affected by OA treatment, honey bees with naturally occurring viruses (in the absence of varroa) that are treated with OA will exhibit lower viral titers compared to untreated honey bees. Additionally, honey bees that are orally inoculated with viruses (in the absence of varroa) and treated with OA will show lower viral titers compared to inoculated

honey bees that do not receive OA treatment. The application of OA at different intervals by the sublimation method in late fall will influence both the efficacy against varroa mites and the overall health of honey bee colonies. If OA is applied at 12-day or 5-day intervals, there will be significant differences in the effectiveness of mite control and colony health. Spreading four OA applications over a longer period (12-day interval) will result in better efficacy than a shorter treatment period (4-day interval). Honey bee colonies treated with OA at these intervals are expected to show better health outcomes compared to untreated control colonies.

The wintering environment influences the effectiveness of OA management strategies on honey bee colony performance. If the impact of OA treatment on colony health is affected by temperature fluctuations post treatment, the same dose of OA will produce different outcomes on colony health in indoor versus outdoor wintering environments.

Chapter 2: Effect of Early Fall Oxalic Acid Trickle Treatment on Virus Levels, Varroa Efficacy and Colony Performance

Abstract

Honey bees have been experiencing high overwintering losses due to stressors such as varroa mites and their associated viruses such as DWV, BQCV, SBV, and IAPV. Oxalic acid (OA) is commonly used to control varroa mites, but its impact on viruses is not well understood especially when applied at times in autumn when efficacy is poor due to the presence of brood in colonies. In this study, we treated 20 colonies once in early autumn with 3.5% OA, while 20 colonies served as untreated controls. We sampled varroa mites pre and post treatment using alcohol washes and sticky boards, and we also sampled honey bees for viruses and protein levels. Additionally, we measured cluster size, colony weight and colony survival. After two weeks, the daily mite mortality of varroa mites increased in the OA treated colonies, however, late fall mean abundance was equivalent in both treatments. Although fall virus levels were not affected by treatment, the levels of DWV-A and SBV viruses were reduced in the spring in treated colonies that survived the winter relative to the untreated controls. Among all the factors tested such as *Nosema*, cluster size, protein, and multiple viruses; only DWV-A and *Varroa* mite levels in the fall significantly predicted colony survival, with higher DWV-A and varroa infestations associated with increased winter mortality.

We further investigated the impact of OA alone on viruses in the absence of varroa mites through cage experiments to determine if OA treatments had direct or indirect effects on virus levels in the

absence of mites. Cages containing bees with low levels of background virus were artificially inoculated with concentrated solutions of either DWV-A or DWV-B viruses in fed syrup in separate experiments. Honey bees in half of these cages were treated with OA at concentrations equivalent to what would occur in colonies during treatments with a trickle formulation, while the other cages remained untreated on were sham treated with the buffer (0.1 M Ascorbic acid and 0.5 M potassium phosphate) that was used for DWV purification. The inoculation was successful in increasing both DWV-A and DWV-B relative to controls but oxalic did not reduce the level of DWV within the 6-7 days period of the experiment. Topical OA treatment reduced the levels of BQCV relative to untreated uninoculated controls in one of the cage experiments. However, the buffer inoculum alone also independently reduced the levels of SBV and BQCV relative to controls.

Key Words: Oxalic acid, Colony health, Varroa efficacy, Deformed Wing Virus (DWV)

Introduction

Honey bees (*Apis mellifera* L.) are vital for pollination of agricultural crops, but their supply to meet those needs are threatened as beekeepers are facing high annual colony losses, especially over winters that make it difficult to maintain colony numbers (Calderone, 2012; Hung et al., 2018). This is of particular concern in North Temperate Climates. According to the Canadian Association of Professional Apiculturists, (CAPA, 2022), overall losses in Canada were 45.5% and in Manitoba up to 57% of colonies died over winter. Honey bee survival over winter is a major concern for beekeepers. While it has been well established that colonies need to be protected from low and variable temperature conditions (Gates, 1914), Varroa (*Varroa destructor* Anderson and Trueman) interacting with other stressors are considered as a major factor responsible for overwintering losses (Currie et al., 2010; Guzmán-Novoa et al., 2010).

Varroa is a globally dispersed ectoparasite (Martín-Hernández et al., 2012; Potts et al., 2010) that feeds on adult and immature honey bees. Varroa has several impacts on colonies that collectively can result in economic damage. Varroa weakens the immune system of honey bees by feeding on the hemolymph and fat bodies (Ramsey et al., 2019). Moreover, Honey bees infested with varroa mites have lower protein levels in haemolymph compared to non-infested bees (G. V Amdam et al., 2004; Weinberg & Madel, 1985). Varroa infestation leads to consequences like a reduction in lifespan in workers, a decrease in colony population, effects on foraging activity, decreased honey production and decreased colony survival (Annoscia et al., 2019; Currie & Gatien, 2006; Nazzi et al., 2012). The combination of varroa and viruses leads to colony losses in all areas of the world where both are found (Dainat et al., 2012b; Francis et al., 2013; Roberts et al., 2020). Virulence of DWV is increased when vectored by varroa, potentially due to a reduction in transmission barrier and weakened immune system in the host honey bee (Annoscia et al., 2019; Nazzi et al., 2012; Wilfert et al., 2016). Before varroa infestation, DWV virus was probably present in many populations at low viral loads, had low prevalence, was largely asymptomatic and was likely transmitted by oral and/or sexual activity. After the introduction of varroa, DWV became more prevalent, at higher levels of abundance and with visible symptoms associated with infections such as deformed wings commonly being expressed (Beaurepaire et al., 2020; Martin & Brettell, 2019). Additionally, the association of varroa with DWV also led to a loss in DWV diversity, which has lead to some variants becoming more prevalent in honey bees (Lanzi et al., 2006; Loope et al., 2019). DWV has three major variants – "A" (Ryabov et al., 2014), "B" (Ongus et al., 2004), and "C" (Mordecai et al., 2016). Of these, DWV-B, is thought to more virulent than previously dominant DWV-A (McMahon et al., 2016; Paxton et al., 2022) however, DWV-A was the first variant associated with colony losses. DWV-B (Martin & Brettell, 2019), was previously named as *Varroa destructor* virus-1 (VDV-1) as it replicates in and is transmitted by varroa (Mordecai et al., 2016). Cage experiments show that DWV-B is equal to or more virulent than DWV-A if more than 107 viral loads are injected to adult

bees (McMahon et al., 2016; Tehel et al., 2019). DWV-C is more recently described; it is also linked with overwintering losses (Kevill et al., 2017; Mordecai et al., 2016). Honey bees inoculated with DWV as immatures show deformed/crippled wings, reduced lifespan and cognitive impairment in the adult stage (Francis et al., 2013). The appearance of large numbers of bees with deformed wings in colonies often marks the final stage before colony collapse resulting from uncontrolled varroa infestations (Ball, 1983; Ball & Allen, 1988; Benaets et al., 2017; Genersch & Aubert, 2010; Sumpter & Martin, 2004; Tentcheva et al., 2004). However, other viruses like BQCV, SBV and Israeli acute paralysis virus (IAPV) further exacerbate honey bee colony decline. BQCV is a common honey bee virus and pathogen for bee queen larvae (Ellis & Munn, 2005; Mondet et al., 2014). It infects all life stages of honey bees: larva, pupa and adult (Y. Chen et al., 2004). It is transmitted both vertically and horizontally (Leach & Drummond, 2018; Tentcheva et al., 2004). Infected queen larva become black in the pupal stage within their cells, which hampers queen replacement, contributing to colony loss (DeGrandi-Hoffman & Chen, 2015; Spurny et al., 2017). Usually, BQCV detected in adult worker bees is asymptomatic (Aubert et al., 2008) but BQCV infects ovaries of queens and honey bee digestive tracts (Y. Chen et al., 2006). BQCV is associated with overwintering losses in some regions (Dainat et al., 2012b; Johnson et al., 2009) but a large study in Canada showed no association with winter survival in either of two years (Borba et al., 2022).

Sacbrood virus is another common virus affecting honey bees particularly their larva (Bailey, 1969; Hitchcock, 1966). It was the first virus identified to be pathogenic to honey bees through its symptoms in larvae (Y. P. Chen & Siede, 2007; White, 1913). Infected larvae form a sac like appearance due to accumulation of ecdysial fluid in their bodies, which ultimately leads to death before pupa formation (Hitchcock, 1966; Nguyen & Le, 2013). Although SBV predominantly affects larva, it can also infect adult honey bees, causing decline in lifespan (Dolezal & Toth, 2018). Infected adult bees however, do not show any clinical symptoms (Anderson & Gibbs, 1989). SBV is transmitted both vertically (from

queen to offspring) and horizontally (infected nurse bee to larvae) (Beaurepaire et al., 2020; De Miranda et al., 2012). SBV cannot replicate in varroa, it only uses varroa as a mechanical carrier (Rosenkranz et al., 2010) but varroa mites play a role in spreading the virus in the colony (Drescher et al., 2017). Pathogenicity of SBV in *Apis mellifera* is less severe in comparison to the Asian honey bee *Apis ceranae (Freiberg et al., 2012)*. SBV, when combined with other stressors like varroa, is associated with colony decline or increased colony death over winter (Borba et al., 2022; Desai & Currie, 2016a).

IAPV is detected in all stages of honey bees, from egg to adult bees, and all castes . This virus affects the nervous system, gut and hypopharyngeal glands (Y. P. Chen et al., 2014). IAPV infected adults show symptoms of paralysis, shivering wings, and disorientation which lead to death (H. F. Boncristiani et al., 2013; Y. P. Chen et al., 2014). Transmission can occur both horizontally and vertically and varroa mites can also spread the infection (Emsen et al., 2015; McMenamin & Genersch, 2015).

Many attempts have been made to control viral diseases, but they have not developed into currently registered products for use in controlling viral infections in honey bees. Researchers have shown RNAi interference can be a promising technology to control IAPV and DWV (Desai et al., 2012; Hunter et al., 2010; Maori et al., 2009). However, destroying infected colonies or managing varroa are currently the most frequently recommended methods to control viral infections (Locke et al., 2017). Deformed wing virus may be more impactful at the colony level than other viruses such as black queen cell (BQCV) and sacbrood (SBV) because the latter two kill the brood before the immature bees and varroa that infect them kill in honey bee cells thus preventing transmission of those viruses among new adult hosts (Sumpter & Martin, 2004). Varroa control in early fall, before the emergence of winter bees, can help reduce viral loads by preventing oral transmission by nurse bees (Locke et al., 2017). (Currie & Gatien, 2006; Francis et al., 2013; Shen et al., 2005).

Varroa control is essential for beekeepers if they are to prevent colony losses and its control typically involves the use of synthetic acaricides or organic chemicals (Mondet et al., 2014). Due to overuse, varroa has developed resistance against many synthetic chemicals like tau-fluvalinate, coumaphos, flumethrin, and amitraz (Currie et al., 2010; Morfin et al., 2022; Rinkevich, 2020; Rodríguez-Dehaibes et al., 2011). Organic chemicals like oxalic and formic acids are seeing increased use as there is currently no evidence of mite resistances developing against them (Tihelka, 2018). Oxalic acid (OA) is found naturally in kiwi, raw legumes, spinach, rhubarb, tea and beet, and varroa is thought to have low chances of developing resistance against it (Maggi et al., 2017; Zayed et al., 2025). Across the last three decades of use, there is no robust evidence of resistance, but it cannot be entirely ruled out (Kosch et al., 2024). Oxalic has been used to treat varroa mite for decades (Popov et al., 1989). Oxalic can be applied to bees by a variety of methods including dissolving it in sugar syrup (Trickling method) or "vaporizing it" directly into the hive (sublimation method) (Charriére & Imdorf, 2002; Van der Steen & Vejsnæs, 2021). Unlike formic acid, OA cannot penetrate capped brood cells, making it most effective during broodless periods when it can only target phoretic mites on adult bees (Charriére & Imdorf, 2002). However, some studies, such as (Parsons, 2017) indicate that early fall OA applications when brood is present can still provide benefits for wintering success, even if they don't significantly reduce varroa mite populations at the time of application.

This unexpected benefit suggests that OA may be helping bees in other ways, either by inhibiting mites from feeding (which could reduce virus transmission) or by directly affecting viruses. Since (Parsons, 2017) study observed these potential benefits using trickle applications in early fall, this study aims to further explore that aspect, investigating whether early fall OA provides protection to bees beyond just killing mites through suppression of viruses or increased total protein in wintering bees. The specific objectives of my study are to investigate the direct and indirect impacts of an early fall OA trickle

treatment on varroa mite when brood is present on viral titers, protein and colony performance in honey bees. Additionally, I aim to assess if there are any direct effects on virus that result from topical application virus inoculated bees in cages that are free from varroa. In addition to direct physiological damage caused by the mite, varroa can transmit viruses while feeding on honey bees or increase susceptibility to viruses like deformed wing virus (Desai & Currie, 2016a; Wilfert et al., 2016). In this study, we focused on several positive sense, single stranded RNA viruses that commonly infect honey bees, including DWV (family Iflaviridae) (Lanzi et al., 2006), BQCV (family Dicistroviridae) (Aubert et al., 2008), SBV (family Iflaviridae) (Li et al., 2019) and IAPV (family Dicistroviridae) (Genersch & Aubert, 2010; Maori et al., 2007). According to (Desai et al., 2016), DWV, BQCV, and IAPV were the most frequently detected viruses across Canadian colonies, with DWV most abundant and strongly linked to colony health, while SBV was more prevalent in unhealthy colonies in Manitoba. Therefore, these viruses were selected due to their widespread occurrence, and known association with *Varroa destructor* and colony decline in Canadian honey bees.

Materials and Methods

Experiment 1- Effect of early fall oxalic acid treatment on efficacy and colony performance

This study was conducted from September 2021 to April 2022. Forty single brood chamber standard Langstroth colonies having ten frames each were located at an apiary site at the Glenlea research station, University of Manitoba (49.6456718420677, -97.15254112927711). Initially, on 30 August, all colonies were assessed to quantify the mean abundance of varroa mites (number of varroa mites per 100 bees) using the alcohol wash method (Gatien & Currie, 2003). Honey bees ~ 250 were collected from the center of the brood chamber in each colony in sample cups and weighed. The bees were then transferred

onto a mesh placed in a plastic box containing 70% ethanol and shaken at 200 rpm for 10 minutes on an Orbit shaker and VWR® Analog Shaker. All mites collected were divided by the estimated number of bees in the sample to calculate the mean abundance. Colonies were assigned to one of two blocks (arbitrarily designated as “low” or “high”) based on the relative mean abundance of varroa mites. The “low” mite block had mite abundances that ranged between 0% to 0.46% (mites per 100 bees), and the “high” mite block had mites that ranged from 0.48% to 9%. Colonies in each block were assigned treatments in a randomized manner. Half of the colonies were assigned to OA treatment in each block, and the remaining colonies were left untreated as controls. The application of OA was carried out on September 8th, 2021, using the trickle method, following the label recommendations. In this procedure, 5ml of 3.5% OA solution in 1:1 sugar syrup was applied per bee space, adhering to the label recommended dose for this region (Lafrenière, 2020).

During the fall period, all the colonies were fed with sucrose syrup (2 parts sugar to 1 part water by volume) *ad libitum* using hive top feeders (Gruszka, 1979). During late fall, when conditions were cooler, colonies requiring supplemental feed were fed with gravity pail feeders. The indoor wintered colonies were later fed syrup by entrance feeders in February, and feeders were replenished weekly according to their requirements.

In preparation for wintering, all experimental colonies were moved to an indoor wintering building on the Fort Garry Campus at the University of Manitoba (49.80886955684351, -97.12749639722962) on November 19th, 2021. On April 27th, 2022, the colonies were transferred out of the wintering building. On April 28th, live colonies were treated with Apivar® strips to kill and quantify any residual mites.

Sample Collection

All the colonies in the yard were sampled before the experiment for alcohol wash, viral titers, and total protein. Samples of approximately 250 bees (used to estimate varroa mean abundance) were collected from the brood nest into sample cups before the application of OA, after treatment application just before wintering, and then once in the spring of the next year (2021-08-30, 2021-11-10, 2022-04-27). Separate collections of worker honeybees were collected in 15 ml falcon tubes for protein and viral titers but were also collected in mid fall as well as on the other dates (2021-08-30, 2021-10-20, 2021-11-10, 2022-04-27). Those samples were immediately placed on ice in coolers in the field and later stored at -80°C upon returning to the laboratory.

Sticky board sampling was also used to record the number of mites falling on the bottom board of the hive following treatments. These boards were prepared manually by spreading Vaseline® on a rigid polyethylene board covered with parchment paper placed on the bottom board of colonies underneath a screened bottom board (Varroa-nators ®). The screened bottom boards prevented bees from falling on the sticky boards but allowed any varroa mites falling from the colony to pass through them and become trapped in the Vaseline®. These parchment papers were later folded and stored individually in small Ziplock bags in a freezer at -15°C until they could be counted. Sticky boards were inserted on 2021-09-08 and replaced at intervals of 2 weeks (2021-09-22, 2021-10-06, 2021-10-20, 2021-11-03) until colonies were moved indoors for winter storage and then monthly while in the wintering building (2021-11-19, 2021-12-20, 2022-01-20, 2022-02-22, 2022-03-18, 2022-04-22). Since the disruption associated with movement of hives could affect the daily mite drop, the sticky boards were also replaced before moving bees from the Glenlea apiary site to the wintering location on the University of Manitoba campus and prior to movement into the wintering building (2021-11-10, 2021-11-12).

Honey bee populations and colony weights were assessed just prior to wintering (2021-11-17) and following removal from the wintering facility in the following spring (2022-04-27). Population size was assessed by viewing the colony from above and below. During cold weather, honey bees cluster tightly together in between the frames and their populations can be estimated based on the number of frame seams (space between adjacent frames) filled. For counting, if the frame seam was filled, it was scored as 1; if it was 3/4th filled, it was scored as 0.75; if the frame seam was half filled, it was scored as 0.5, and so on. The average of top and bottom scores were used as the cluster size (Chabert et al., 2021). Colonies had inner covers with a top entrance for ventilation during indoor storage. In fall and spring the colonies were weighed using a platform scale. The final spring weight was adjusted by subtracting the weight of any additional syrup fed during winter from the total weight in spring. The amount of food consumed when bees were held within the wintering building was used to estimate when colonies died during winter by dividing the amount of food consumed by the number of frames of bees in fall and the average daily food consumption (0.015 Kg per frame) that was obtained from load cell data inside the wintering building (unpublished data) to provide the estimated days the colony was expected to have survived. These data were subsequently used for principle component analyses.

On April 27th, 2022, the colonies were transferred out of the overwintering building and sampled as described above. After sampling adult bees, any live colonies were treated with Apivar® strips on April 28th, to kill any remaining mites. During this 56-day treatment (2022-04-28, 2022-06-22), sticky boards were utilized to count the number of mites that fell from the hive. This was to estimate the total mite populations remaining in the hive at the end of the experiment so it could be added to those that fell during the experiment and thus estimate the total mite population in the hive.

Experiment-2- Effect of OA on viruses in the absence of varroa mites

Preliminary trial and dosage evaluation

Preliminary trials were first conducted in June 2021 to determine the concentration of OA, that would reduce the number of varroa mites without killing all of them within the short period of the assay to emulate efficacy results from OA field trials (Parsons, 2017). In this experiment OA concentrations of (2%, 3%, 4%, 5%, 7%, 8%, and 10%) (weight/volume) were administered to bees in a 1:1 sugar syrup. These OA solutions were then trickled on caged bees that had been infested with varroa mites. For these trials, the OA was pipetted on individual bees to carefully control the dosage each bee received.

For these experiments, plexiglass cages (10.8 cm height, 15.3 cm width, and 7.7 cm depth) were used. These cages had two feeder holes on the top surface fitted with one tube containing water and the other 1:1 sugar syrup (by volume). The feeder tubes were covered with muslin cloth and secured with a rubber band, which prevented the water and syrup from freely flowing into the cages but allowed access by the bees. A piece of plastic window screen was hung vertically in the cage to help bees reach the feeder tubes (Figure 2. 1).

Varroa mites were collected from colonies maintained in a separate mite rearing yard at U of M. To collect mites, bees were scooped into Mason jars with mesh lids then anesthetized by sealing the Mason jar in a Ziplock bag and filling the bag with CO2 until all bees ceased movement. Anesthetized bees were then transferred into a plastic box with a hardware cloth mesh bottom and provided with a constant supply of CO2 at 5 L/min (Currie & Tahmasbi, 2008). Bees were shaken for 5 minutes at 400 rpm to detach the varroa mites from the honey bees and they were collected on a moist paper towel in a petri dish using a small paint brush. Petri dishes and the mites were allowed to feed on pupae added to the dish then stored in an incubator overnight prior to introduction in cages on the next day. For OA

treatment, honey bees from mite-free colonies were anesthetized as described above. OA was applied to the anesthetized bees by pipetting 0.41µl of weight/volume (2%, 3%, 4%, 5%, 7%, 8%, and 10%) OA solution onto each honey bee in separate cages.

After pipetting the OA sugar solution onto each bee, the workers were transferred to the bioassay cages with 150 bees added to each cage. Mites were placed on bees within cages using a paintbrush with 10-15 mites added in each cage (150 bees). The mites were added to cages with equal numbers in each treatment but the total number varied according to the availability of live mites since there were fewer mites available in June and some mites died overnight in the incubator. Cages with bees and mites were kept in incubators for 24 hours at 32° C. A dish of water was placed in the chamber to maintain humidity. Additionally, every cage within the incubator was placed on paper lined with petroleum jelly to trap any mites escaping from the cages so they could be quantified and to prevent any potential cross contamination among cages. After 24 hours, the number of dead mites was counted, and the percentage mortality was calculated. We found 2% OA to be suitable for the cage experiment as it produced a level of mortality similar to that found in field trials in our lab with early fall oxalic applications (Parsons, 2017).

Experiment 2.1- Experimental Assessment of DWV-B Inoculation and 0.41 µl of 2% Oxalic Acid Treatment in Honey bee Cages

Deformed wing virus-B (DWV-B) was extracted from adult bees collected from brood nests of colonies that had high levels of varroa and contained workers with visible deformed wings symptoms (colonies were located on campus at the University of Manitoba). Purified virus was propagated by injecting virus from infected bees into pupae collected from isolated Australian bees (described below) according to protocols described in (A. Chapman, 2024; Desai et al., 2012). Extracted virus used in the

cage trial for experiment 2.1 consisted of 96.68% DWV-B, 3.07% DWV-A, 0.20% IAPV, 0.02% BQCV, and 0.02% SBV.

To obtain colonies for cage trials that would be expected to have low background levels of DWV we used bees imported from Australia which were varroa-free at the time of the experiment and maintained in secluded yards to prevent varroa infestation from neighboring colonies. Upon arrival in Canada, Australian packages were hived onto frames with fresh foundation in an isolated bee flight room at the U of M campus and then transferred to an isolated apiary to allow us to collect workers for experiments. Workers were collected from October 15th, 2021, to October 22nd, 2021. Bees were lightly anesthetized with carbon dioxide to allow them to be sorted into cages. A total of 22 cages were used; each cage received 21 g (approx. 150 bees) of honey bees. After transferring honey bees,11 cages were treated with OA by pipetting 0.41µl of 2% OA in 1:1 sugar syrup per bee as described above. In this study, 20 cages were randomly assigned to two groups of 10 cages (10 OA treated and ten no-oxalic/no buffer/control cages). Half of the cages from each group were randomly assigned to be orally inoculated with the deformed wing virus as described below, and the other half were not inoculated. Additionally, two supplementary cages were fed with only the buffer solution but received no additional DWV inoculation. One of these cages underwent OA treatment, while the other remained untreated.

For oral inoculation of DWV-B, honey bees were fed purified virus mixed with enough 1:1 sugar syrup to allow the entire amount to be consumed within the first day of the trial (7.5 ml). When that syrup was all consumed, they were supplied with non-inoculated sugar syrup *ad libitum*. Bees were inoculated at a rate that would provide at least 10^8 viral genomes/bee which is enough to establish infection (de Miranda et al., 2013). Thus, for each cage of 150 bees we fed $1.5*10^{10}$ viral genomes. Extraction buffer (0.1 M Ascorbic acid and 0.5 M potassium phosphate) was used to dilute the original concentration of viral titers to achieve this dose and 8.62 µl of virus in buffer were mixed with the syrup fed on day 1

(7.5ml). For cages that did not consume the initial dose on day one, bees were allowed to consume all the virus-infested syrup (up to 48 hours) after which tubes were topped up to 15 ml with fresh sugar syrup daily.

Sample collection

Cages were monitored every day to collect the dead bees which were removed. Data for the daily mortality of bees was recorded. On the last day of the experiment (22 Oct 2021), all the live bees were removed and stored at -80° C. Live and dead bee samples were subsequently analyzed in the lab to determine the level of 5 economically important viruses (DWV-A, DWV-B, IAPV, BQCV, SBV). Bees were homogenized with Spex™ SamplePrep Geno/Grinder 2010 and a Qiagen RNeasy® Mini kit was used for RNA extraction, and cDNA was prepared by using iScript™ cDNA Synthesis Kit. The last step was to do real-time qPCR using CFX96™ Real-Time System (Bio-Rad™) to quantify viral titers. Virus levels were done in triplicate and compared against a standard curve using g-blocks.

Experiment 2.2- Experimental Assessment of DWV-A Inoculation and 4.1µl of 3% Oxalic Acid Treatment in Honey bee Cages

The experiment was repeated using DWV-A (99.9% pure), from September 28th, 2022, to October 4th, 2022. Methods were similar to experiment 2.1 but, in addition to using a different virus strain this experiment used cages that were treated with a higher amount of OA per bee and bees were inoculated with a higher dose of virus. DWV-A virus was produced as described above for DWV-B, but virus was obtained from colonies from other studies in our laboratory that were identified as being primarily infected with DWV-A. The volume of diluted virus required for oral inoculation ($1.16*10^9$ µg/µl) was 5.18 µl per cage. Virus was added to 3ml of syrup to allow most of the syrup to be consumed by the 60 bees in 24 hours. Additionally, ten cages were fed on extraction buffer with no virus as an

additional sham control. In these cages, 5.18 µl of buffer was added to 3 ml of syrup. There were five control cages, which were fed on syrup. The amount of syrup containing OA used for treating individual bees in this experiment was 4.1 µl of 1:1 sugar syrup per honey bee at 3% OA. Twenty five cages were used, and each cage was filled with 60 Australian honey bee workers. The cages were divided into 5 groups having 5 cages each: 10 cages treated with OA and 15 control cages: 1-five cages were treated with OA and orally inoculated with DWV-A virus mixed with buffer; 2. five cages were treated with OA and orally fed with buffer; 3. five control cages were orally inoculated with DWV-A virus mixed with buffer, 4. five control cages were orally fed with buffer and 5. five control cages without inoculation with buffer or virus.

Sample collection

In this experiment, only live bees were processed to quantify post-treatment virus levels. A pre-experiment sample of worker bees was collected to assess background levels of virus, and live bees were collected at the conclusion of the experiment on the sixth day. Dead bees were removed from cages each day and quantified but not used for virus analysis. These sample bees were analyzed to quantify DWV-A, DWV-B, BQCV, and SBV as described above. We did not analyze IAPV post treatment in this experiment as it was present in only trace amounts in the pre-experiment sample.

Statistical Analysis

For experiment 1, data were analyzed using repeated measures analyses of variance where treatment and mite level (mean abundance prior to treatment) were main effects, the colony (hive) was the experimental unit, and the date of sampling was a repeated measure (proc mixed, online SAS studio 2023). Data were checked using the Shapiro-Wilk test for normality and Levene's test to assess homogeneity of variance. Where data did not meet the assumptions for ANOVA, they were transformed to

improve normality and/or homogeneity of variance. Each response variable was run under different covariance structures, and the one with the lowest AIC was selected. Where significant differences among treatments were found, post hoc tests were performed with (Bonferroni $P < 0.05$) adjustment. In cases where significant interactions were found with date, a separate Analysis of Variance (Proc mixed) was performed for each date. November data was analyzed separately to see the effect of treatment before wintering. Mean abundance data were arcsine transformed prior to analyses and data were analyzed using an autoregressive heterogeneous “arh(1)” covariance structure. The total protein data were log transformed prior to analysis and data were analyzed using a compound symmetry “cs” covariance structure.

The virus data was also log-transformed. A random effect was virus within hive ‘(random virus (hive)’, it was specified since multiple viruses were tested within each colony in the fall (August, October, and November). Spring data were analyzed separately due to high levels of colony mortality over winter. Different covariance structures were selected when examining individual viruses. The data for BQCV underwent analysis utilizing an autoregressive 'ar(1)' covariance structure. For DWV-B, a compound symmetry 'cs' covariance structure was employed. In the case of SBV, the analysis utilized the variance components 'vc' covariance structure. The covariance structure used for DWV-A and IAPV was autoregressive heterogeneous ‘arh(1)’.

Data for cluster size were square root transformed. As many colonies died in spring, the data was analyzed by deleting colonies with zero cluster size. Cluster size data were analyzed using an unstructured “un” covariance structure. The colony weight data were log-transformed and analyzed using an unstructured “un” covariance structure. As many colonies died in spring, we analyzed only alive colonies using the “un” covariance structure.

The values for mite drop on each sampling period were converted to a daily mite mortality rate (DMMR) (based on the total number of mites in the colony at the beginning of the experiment) and adjusted for the number in the prior sampling period to account for variations mite level among hives that could affect daily mite drop. The mortality rate for each date was calculated by dividing the value on the sticky board by the total mite count in the previous period (sum of mite count from beginning date to previous date). Finally, the DMMR was obtained by dividing the mortality rate by the number of days the sticky board was placed inside the hive. The mite drop data were analyzed for the dates up until 2021-11-10 when the colonies were transported to the honey house for wintering. The DMMR was transformed using arcsin (arsin (sqrt (DMMR)) *57.3) transformation. Data were analyzed using an "un" covariance structure.

For the "Early Fall" experiment, colony survival data were analyzed using binary logistic regression (proc logistic) to evaluate the effects of virus levels, *Varroa destructor* infestation, and colony level factors on the likelihood of colony mortality. The response variable was colony status (alive or dead), and the colony (hive) was considered the experimental unit. Predictor variables included log-transformed virus titers (DWV-A, DWV-B, BQCV, SBV, and IAPV), log-transformed colony weight, protein content, and cluster size, and arcsine square root transformed *Varroa* infestation levels. The variables treatment, varroa block, and Nosema infection status were included as categorical class effects. A backward stepwise model selection approach was used to retain only significant predictors in the final model. Odds ratios and confidence intervals were used to evaluate the direction and strength of associations between predictor variables and the probability of colony death. Virus data from November only were used in this analysis to evaluate conditions at the onset of winter. All transformed variables were assessed for multicollinearity prior to inclusion in the final model.

For multivariate exploration of the relationships among OA treatment, *Varroa destructor* infestation, colony level factors, and virus levels, principal component analysis (PCA) was conducted using OriginPro (OriginLab Corporation, Northampton, MA, version [2023]). Variables included in the PCA were OA treatment, fall mite levels (mites per 100 bees), cluster size, colony weight, overwinter survival, and virus titers for DWV-A, DWV-B, BQCV, SBV, and IAPV. Prior to analysis, all continuous variables were standardized using z-score transformation to ensure equal scaling and to meet the assumptions of PCA.

PCA was performed using the correlation matrix method. The analysis generated eigenvectors and eigenvalues for eight principal components. The first two principal components (PC1 and PC2) accounted for 19.87% and 14.93% of the total variance, respectively, and were retained for interpretation due to their higher explanatory power and biological relevance.

For cage trials (Experiment 2), data were analyzed using mixed model ANOVA (repeated measures design), where treatment, was the main effect, cage was the experimental unit (subject), and repeated samples were processed on each of the selected viruses within each cage. Gene copies for each virus were transformed using log10(x+1) prior to analyses. The most appropriate covariance structure with the lowest AIC was selected after comparing different covariance structures. For experiment 2.1 a 'variance component' covariance structure was used. Single degree of freedom contrast statements were used to compare different combinations of treatments. For experiment 2.2, data were analyzed using an "un" covariance structure. Where significant differences among treatments were found, post hoc tests were performed with (Bonferroni $P < 0.05$) adjustment.

Results

Experiment 1 results

Mean Abundance of Varroa Mites

Early fall applications of OA resulted in a significant effect of treatment ($F = 4.25$, $df = 1, 36$; $p = 0.0465$) prior to wintering; the average mean abundance of varroa mites was significantly higher in control colonies (5.37) than in the treated colonies (3.31). When looking within the dates, there was no effect of treatment.

During the fall season, there was significant variation in mite abundance with date ($F = 86.27$, $df = 1, 36$; $p < 0.001$), varroa mite abundance significantly increased over time from 1 September (1.1247) to 10 November (7.5565), even in OA-treated colonies. Additionally, there was a significant initial mite block*date interaction ($F = 11.37$, $df = 1, 36$; $p = 0.0018$) Figure 2. 2. To explore this interaction we did a separate analysis by date. As anticipated, in September, the mean abundance of varroa in the high block was significantly higher than in the low block (($F = 56.03$, $df = 1, 36$; $p<0.0001$) ($F = 4.47$, $df = 1, 36$; $p = 0.0414$) . However, in November, mite levels that differed between blocks in September were equivalent ($F = 0.34$, $df = 1, 36$; $p = 0.5647$) . In spring, following indoor wintering, only surviving colonies were analyzed and there was no significant difference between the high and low initial mite blocks ($F = 1.54$, $df = 1, 2$; $p = 0.3400$). Additionally, in spring, the average mean abundance of varroa mites did not differ among surviving control colonies (3.88) and treated colonies (5.35) ($F = 0.40$, $df = 1, 2$; $p = 0.5922$).

Sticky board data

For DMMR, there was an interaction between oxalic treatment and date ($F = 6.08.$, $df = 4, 180$; $p=0.0001$). The oxalic treated hives had higher DMMR after two weeks, indicating significantly more

mites killed by the oxalic treatment (Figure 2. 3). After the first sampling period there were no effects of oxalic on DMMR but there was significant variation among dates with mite mortality rate increasing with the date ($F = 44.21.$, $df = 4, 180$; $p<0.0001$). There was no effect of the initial mite level block on the DMMR ($F = 0.91.$, $df = 1, 180$; $p=0.3416$).

Cluster Size

In colonies that were alive in spring, neither oxalic treatment nor initial mite block showed significant effects on cluster size. Cluster size in live colonies was marginally reduced ($F = 16.43$, $df = 1, 2$; $p = 0.0558$) in the spring season (2022-04-27) in comparison to fall (2021-11-17) (Figure 2. 4**)**.

Colony Weight

In colonies that were alive in spring, neither treatment nor initial mite block showed significant effects on colony weight. Colony weight decreased ($F = 258.68$, $df = 1, 36$; $p < 0.001$) in the spring season (2022-04-27) in comparison to fall (2021-11-17) (Figure 2. 5).

Total Protein

There was no overall effect of oxalic treatment, mite block or interaction between oxalic treatment and initial mite level. Total protein in workers did vary with Date ($F = 39.15.$, $df = 3, 72$; $p < 0.001$). Bees had significantly higher levels of total protein in September (2021-09-1) than in Nov (2021-11-10) and April (2022-04-27). Although the worker protein level increased dramatically in November compared to October (2021-10-11). There was no significant difference in protein levels in workers from surviving colonies in April when compared to bees from colonies collected in October or November (Figure 2. 6).

Viruses

During the fall period of 2021, neither the early oxalic treatment nor the mite level block resulted in significant main effects on virus particle abundance ($P > 0.05$) for any of the viruses tested (DWV-A, (DWV-B), (BQCV), (IAPV), or SBV). All viruses except IAPV showed significant variation in date but with different patterns that were somewhat unique to each virus. For DWV-A ($F = 86.09$, $df = 2, 72$; $p < 0.0001$), its level increased from August (2021-08-30) to October (2021-10-11) and then to November (2021-11-10). For BQCV ($F = 9.24.$, $df = 2, 72$; $p=0.0003$), the level of virus decreased significantly from August to October and then increased from October to November. However, there was no significant difference between virus levels in August and November. For SBV, ($F = 16.64.$, $df = 2, 72$; $p<0.0001$). virus levels were lower in October and November than in August, but there was no difference between levels in October and November. For DWV-B, there was a significant effect of the Date ($F = 125.90$, $df = 2, 72$; $p < 0.0001$); DWV-B significantly increased from August to October and then to November. Treatment did not affect DWV-B. Variation of all the virus levels with date is shown in Figure 2. 7.

When the spring data was analyzed, initial mite infestation blocks from the previous fall had a long-term effect on virus levels in spring for DWV-A and BQCV. Colonies with a higher initial mite infestation in September that survived winter had lower DWV-A the following spring than the colonies with the lower initial mite block (DWV-A; $F = 160.30.$, $df = 1, 2$; $p=0.0062$) (Figure 2. 8). For BQCV, colonies with the higher initial mite levels in September had higher BQCV levels from than from those in the low initial mite block (BQCV; ($F = 160.30.$, $df = 1, 2$; $p=0.0062$) (Figure 2. 8**)**.

There was also a significant effect of early fall OA treatment on virus levels in colonies that survived to spring but only for DWV-A and SBV. For both DWV-A ($F = 173.64$, $df = 1, 2$; $p<0.0057$) and

SBV (F = 17.46., df = 1, 2; p=0.0528) , the surviving oxalic treated colonies had lower virus levels than the controls (Figure 2. 9).

Colony Survival

For the early fall field experiment, backward stepwise logistic regression identified the most significant predictors in fall related to survival of colonies to the following spring, were DWV-A and v*arroa* infestation levels (Figure 2. 10). Variables such as colony weight, fall cluster size SBV, BQCV, nosema infection, protein content, IAPV, and DWV-B, were removed from the model during backward selection due to non-significance ($p > 0.17$). DWV-A significantly affected colony survival ($p = 0.0062$), with colonies experiencing higher DWV-A levels having reduced survival chances. The odds ratio for DWV-A was 0.592, indicating that for each unit increase in log-transformed DWV-A, the odds of colony survival decreased by approximately 41%. Similarly, *Varroa* infestation also significantly influenced survival ($p = 0.0337$), with an odds ratio of 0.770, suggesting that higher v*arroa* infestation reduced the likelihood of colony survival.These findings were further supported by the principal component analysis (PCA), which showed a strong positive association between OA treatment, BQCV and predicted survival time of colonies, and negative associations of survival with both fall varroa mite levels and DWV-A viral loads (Figure 2. 11). In the plot PC-1 is associated oxalic (0.3057) and survival (0.5742) accounting for 19.0% of the variation while PCA-2 is more strongly associated with DWV-A (-0.3415) and Fall mites (-0.2211) and accounted for 14.9% of the variation. The PCA loading plot revealed that survival and OA treatment vectors aligned closely, while DWV-A and late fall varroa mite levels pointed in the opposite direction with fall mite level having a stronger influence than DWV-A.

Experiment 2 results

Experiment 2.1

Cages were treated with 4 different treatments. First was no virus inoculum and no oxalic, second was no virus inoculum and +oxalic treatment, third was DWV-B inoculum and no oxalic and fourth was DWV-B inoculum and oxalic treatment (Figure 2. 12). There was an overall effect of treatment ($F=62.05$, $df=3,16$, $p<0.0001$) when pooling all viruses. Orthogonal contrasts showed that inoculated cages had higher viruses than non inoculated cages ($F = 177.07.$, $df = 2, 16$; $p<0.0001$) for both oxalic treated ($F = 55.89.$, $df = 1, 16$; $p<0.0001$) and no oxalic cages ($F = 128.65.$, $df = 1, 16$; $p<0.0001$). Overall, there was no effect of oxalic on viruses ($F = 1.60.$, $df = 1, 16$; $p=0.2245$). However, within no inoculum cages, the oxalic increased background levels of viruses in comparison to no-oxalic cages ($F = 7.99.$, $df = 1, 16$; $p=0.0122$). Within inoculated cages, oxalic did not increase or decrease virus levels ($F = 1.08.$, $df = 1, 16$; $p=0.3139$).

Overall, there were significant differences in the levels of different viruses in cages ($F=46.62$, $df=4,64$, $p<0.0001$) (Figure 2. 12). As expected, the virus that bees were inoculated with DWV-B (5.1824) had the highest genocopies, followed by BQCV (4.2215), DWV-A (3.6424), IAPV (3.2862), and then SBV (1.8678). All viruses were significantly different from each other except DWV-A, which had similar levels to BQCV and IAPV.

There was significant interaction between virus and the various treatments ($F=7.09$, $df=12,64$, $p<0.0001$) Figure 2. 12. When analyzed by virus, for DWV-A, different treatments affected DWV-A levels ($F = 15.25$, $df = 3, 16$; $p < 0.0001$) however, the oxalic applications did not affect levels of that

virus in worker bees relative to controls (Figure 2. 12). Interestingly, despite very low levels of DWV-A in the original inoculum, feeding it to workers significantly increased DWV-A, in comparison to non-inoculated cages ($F = 42.48$, $df = 1, 16$; $p < 0.0001$) (orthogonal contrast). In non-oxalic cages, inoculated cages (5.3074 $\pm$ 0.4706) had higher DWV-A than non-inoculated cages (1.5220 $\pm$ 0.4706) ($F = 32.35$, $df = 1, 16$; $p < 0.0001$) (orthogonal contrast). Similarly, within the two oxalic-treated cage groups, inoculated cages (5.0447 $\pm$ 0.4706) had higher DWV-A than non-inoculated cages (2.6955 $\pm$ 0.4706) ($F = 12.46$, $df = 1, 16$; $p = 0.0028$) (orthogonal contrast).

The different treatments affected DWV-B concentrations but not as expected ($F = 146.28$, $df = 3, 16$; $p < 0.0001$). Overall, oxalic cages had higher virus levels than non oxalic cages ($F = 4.34$, $df = 1, 16$; $p=0.0537$) (orthogonal contrast). When looking within in non-inoculated cages, OA treated cages (2.8798 $\pm$ 0.1859) had higher DWV-B viral loads than non-oxalic cages (3.6263 $\pm$ 0.1859) ($F = 8.06.$, $df = 1, 16$; $p=0.0118$) (orthogonal contrast). On the other hand, when looking within inoculated cages, there was no significant difference within oxalic (7.1257 $\pm$ 0.1859) and no-oxalic cages (7.0979 $\pm$ 0.1859) ($F = 0.01.$, $df = 1, 16$; $p=0.9173$) (orthogonal contrast).

As expected, inoculum significantly increased DWV-B in comparison to non-inoculated cages ($F = 430.76.$, $df = 1, 16$; $p<0.0001$) (orthogonal contrast). In non-oxalic cages, inoculated cages (7.0979 $\pm$ 0.1859) had higher DWV-B than non-inoculated cages (2.8798 $\pm$ 0.1859) ($F = 257.37.$, $df = 1, 16$; $p<0.0001$) (orthogonal contrast). Similarly, in oxalic-treated cages, inoculated cages (7.1257 $\pm$ 0.1859) had higher DWV-B than non-inoculated (3.6263 $\pm$ 0.1859) ($F = 177.3$, $df = 1, 16$; $p<0.0001$) (orthogonal contrast).

For BQCV, there was no significant treatment effect ($F = 1.92$, $df = 3, 16$; $p = 0.1675$). However, for SBV, treatment had a significant effect ($F = 11.80$, $df = 3, 16$; $p = 0.0002$); inoculum significantly

increased SBV in comparison to non-inoculated cages ($F = 31.27.$, $df = 1, 16$; $p<0.0001$) (orthogonal contrast). In non-oxalic cages, inoculated cages (3.0429 $\pm$ 0.2809) had higher viruses than non-inoculated cages (0.9753 $\pm$ 0.2809)] ($F = 27.09.$, $df = 1, 16$; $p<0.0001$) (orthogonal contrast). In oxalic cages, inoculated cages (3.0429 $\pm$ 0.2809) also had higher viruses than non-inoculated cages (0.9753 $\pm$ 0.2809)] ($F = 7.31.$, $df = 1, 16$; $p=0.0156$) (orthogonal contrast), however, oxalic treatment did not affect viral concentrations relative to controls ($p > 0.05$)

For IAPV, different treatments had a significant effect ($F = 7.58$, $df = 3, 16$; $p = 0.0023$); inoculum significantly increased IAPV in comparison to non-inoculated cages ($F = 20.73.$, $df = 1, 16$; $p=0.0003$) (orthogonal contrast). In non-oxalic cages, inoculated cages (4.6197 $\pm$ 0.5397) had higher IAPV concentrations than non-inoculated (1.5292 $\pm$ 0.5397) ($F = 16.39.$, $df = 1, 16$; $p=0.0009$) (orthogonal contrast). In oxalic cages, inoculated cages (4.4104 $\pm$ 0.5397) also had higher IAPV viruses than non-inoculated (2.5857 $\pm$ 0.5397) ($F = 5.71$, $df = 1, 16$; $p=0.0295$) (orthogonal contrast) however, oxalic treatment did not affect viral concentrations ($p > 0.05$).

Experiment 2.2

In this experiment cages received 5 different treatments: 1. no inoculum and no oxalic , 2. DWV-A inoculum and no oxalic, 3. DWV-A inoculum and oxalic, 4. buffer, no inoculum and no oxalic and 5.buffer, no inoculum and +oxalic. Overall, there were significant differences in the pooled levels of different viruses in cages ($F=13.02$, $df=3,60$, $p<0.0001$).When overall virus levels were compared across all treatments including both inoculated and non-inoculated cages then, DWV-A (2.7799 $\pm$ 0.2734) had the highest average gene copies but was not significantly different from BQCV (2.1089 $\pm$ 0.2734), or DWV-B (2.0262 $\pm$ 0.2734). However SBV (0.8558 $\pm$ 0.2734) had lower average gene copies than the other three viruses ($p <0.05$; Bonferroni).

There was no overall effect of oxalic treatment (F=2.3, df=4,20, p=0.0947) on virus concentration, however there was interaction between virus and treatment (F=4.75, df=12,60, p<0.0001) (Figure 2. 13). For DWV-A, different treatments affected virus levels (F = 5.32, df = 4, 20; p = 0.0044); as expected inoculation with DWV-A significantly increased DWV-A in comparison to non-inoculated cages (F = 16.70., df = 1, 20; p=0.0006) (orthogonal contrast). In no oxalic cages, cages inoculated with DWV-A (4.2450 $\pm$ 0.7791) showed a significant increase in DWV-A gene copies in comparison to non-inoculated cages (1.7628 $\pm$ 0.7791) (F = 5.07., df = 1, 20; p=0.0357) (orthogonal contrast). Similarly, in oxalic treated cages, cages inoculated with DWV-A (5.1678 $\pm$ 0.7791) showed a significant increase in DWV-A gene copies in comparison to non-inoculated cages (1.2822 $\pm$ 0.7791) (F = 12.44., df = 1, 20; p=0.0021) (orthogonal contrast). However, oxalic did not affect DWV-A within inoculated or uninoculated groups. (p >0.05). For DWV-B, treatments had no significant effect on virus concentrations (F = 1.32, df = 4, 20; p = 0.2952). For BQCV, different treatments affected virus level but not as anticipated (F = 4.25, df = 4, 20; p = 0.0119). OA treated cages had lower level of viruses than controls (F = 5.74, df = 1, 20; p = 0.0265) (orthogonal contrast). Additionally, honey bees fed with buffer as one of the controls (1.9304 $\pm$ 0.1598) showed significantly lower BQCV levels than no buffer colonies (2.6464 $\pm$ 0.1598) (F = 10.04, df = 1, 20; p = 0.0048) (orthogonal contrast). Inoculum did not affect the level of BQCV (F = 0.01 df = 1, 20; p = 0.9259) (orthogonal contrast)

Different treatments also had a significant effect on SBV levels (F = 4.54, df = 4, 20; p = 0.009); honey bees fed with buffer as one of the controls (0.8101 $\pm$ 0.2844) showed significantly lower SBV levels than no buffer colonies (1.8423 $\pm$ 0.2844) (F = 6.59, df = 1, 20; p = 0.0184) (orthogonal contrast). Inoculum did not affect the level of SBV (F = 2.23 df = 1, 20; p = 0.1507) (orthogonal contrast) and oxalic did not affect the concentration of SBV (p < 0.05).

Discussion

This study aimed to examine the effects of early fall applications of the oxalic acid (OA) trickle method on varroa and viruses in honey bees under both field and laboratory conditions. In the field experiment, one application of 50 mL of 3.5% OA was applied in early fall in the presence of brood and the results were compared with untreated control colonies. OA lowered the DMMR of varroa mites in the first two weeks relative to controls but did not affect their mean abundance at any time nor did it affect measures of late fall cluster size, colony weight or protein levels. The field experiment demonstrated OA resulted in a reduction in DWV-A and SBV but not other viruses and did so only in colonies that survived to spring. Fall mite levels and DWV-A were negatively associated with colony survival and predicted survival time. Although OA did not improve colony survival in this study it was positively associated with predicted survival time.

In follow up cage experiments, we investigated the impact of OA on viruses alone without the influence of varroa mites since mites were at equivalent levels in treated and control colonies in the field experiment.. When caged bees were inoculated with DWV-B or DWV-A . The OA treatments had mixed effects on levels of viruses. Oral inoculation with DWV-B and trace amounts of other viruses increased levels of DWV-A, DWV-B SBV and IAPV but not BQCV. However, inoculation with DWV-A and trace amounts of other viruses increased only levels of DWV-A relative to uninoculated colonies. In one experiment OA reduced BQCV levels relative to untreated bees. However, the buffer alone or in combination with oxalic both also contributed to a reduction in BQCV levels. Feeding buffer alone caused a reduction in SBV relative to controls in caged bees.

Colonies treated with a solution of 3.5% OA in sugar syrup trickled on bees in early September showed higher DMMRs two weeks after treatment, indicating some short-term efficacy of OA in

mitigating mite populations even though substantial amounts of brood were still present in colonies. However, DMMR was impacted with oxalic treatment only for a short period and the mean abundance of varroa mites later in fall did not remain suppressed by the early fall OA treatments. The mean abundance of varroa mite populations increased from August to November, in all colonies including in colonies treated with OA. Our study applied OA in early September, a period typically associated with declining but still significant brood rearing activity in temperate climates (J. L. Harris, 2008) and continued monitoring until the following spring. Brood levels were not quantified in this experiment, but the continued increase in mite levels in treated colonies in the period when brood rearing tapers off in our region is typical (Gatien & Currie, 2003) and despite efforts to minimize drifting of bees some reinfestation of treated colonies may have occurred as both treated and untreated colonies were held within the same apiary. Similarly, treatment effects associated with, the number of mites a colony had in September influenced changes over the fall and did not differ by November. These findings suggest that in terms of efficacy against varroa a single OA trickle treatment during periods of active brood rearing in fall provides only limited and short-lived benefits, and is insufficient to suppress population growth of v*arroa* mites into winter. These results align with previous findings showing that OA does not provide effective long term protection when brood is present (Berry et al., 2022; K. C. Evans et al., 2022; Gregorc & Planinc, 2001; Jack et al., 2020; Parsons, 2017). Rademacher & Harz (2006) reviewed studies that applied 3.5% OA trickle treatments during broodless periods and showed substantially higher mite reduction than treatments with brood present. Even multiple treatments of 3.5% OA applied in colonies containing brood showed only 36% efficacy (Schuster & Schürzinger, 2003). In contrast, (Charriere, 2004) and (Moosbeckhofer, 2001) observed over 95% efficacy with single application of 3.5% OA trickle during broodless conditions. Similarly, Nasr et al., (2001) reported that 90% efficacy was achieved with single application of 3.5% OA trickle in late fall with limited brood in Canada.

In spring, only surviving colonies were sampled and thus varroa levels would be expected to be influenced by any factors that in combination would affect survival to eliminate colonies with higher mite levels. Mite levels across all groups were similarly low, and no significant differences remained between colonies that were treated with early fall OA or with those that had started the fall with high or low infestations. Overwintering outcomes were likely not determined solely by fall mite levels, other factors such as colony strength, fall colony weight, nosema level, virus load, protein levels or long term influences associated with treatment played a role in shaping, survival and spring infestation levels and pathogen loads (discussed below). Overwintering survival can be increased by ensuring varroa mite levels are low in fall, and viral loads are reduced before winter brood emergence (Currie & Gatien, 2006; Desai & Currie, 2016a; Van Dooremalen et al., 2012).

In this study, both varroa abundance and the levels of DWV-A and DWV-B levels all increased over time, particularly from August to November. This trend was observed for both treated and untreated colonies. Since varroa is a vector of DWV (Genersch & Aubert, 2010), ineffective mite suppression can lead to increases in virus replication and spread within colonies. Although we did not observe effects of oxalic treatment on any virus levels in fall sampling, the use of 3.5% OA trickle in September showed long term effects on viruses levels detected in colonies that survived in April. Oxalic treated colonies had diminished DWV-A levels and marginally lower SBV levels relative to untreated colonies. This "long term effect" of oxalic in spring could be a residual effect associated with the slight reduction in mite levels at a critical time in fall when winter bees are being produced, differential survival of colonies with mixed pathogen loads or a more direct effect of oxalic on suppressing virus, an aspect that we studied further in cage trials. It should be noted that colony survival was very low in spring after an abnormally long period of winter confinement so treatment differences we observed should be viewed cautiously and further studies in a year with better winter survival would be needed before recommendations on the

value of September treatments could be made. The early oxalic treatment and its effect on initial mite level did not significantly affect overall virus levels in November; nevertheless, temporal variations in other viruses were noted unassociated with oxalic treatment. In contrast to DWV, BQCV levels diminished from August to October and subsequently rose in November. SBV levels were diminished in October and November relative to August. Whereas IAPV levels remained the same throughout the sampling period.

The effects of OA on virus levels in bees varied between field and cage experiments. It should be noted that our treatments with a trickle formulation of OA applied to full size colonies or to bees within the cages used different doses and concentrations of OA. However, the cage studies were designed with an attempt to approximate realistic field exposure rates. The OA applications showed mixed impacts on different viruses in the caged "varroa free" bees. In our first cage experiment trial we used an inoculum consisting primarily of DWV-B and applied 0.41 µl of 2% oxalic (which was identified as an appropriate dose in a preliminary trial that showed efficacy against mites). This dosage of oxalic showed unexpected results for cages having natural levels of several viruses, in that it marginally increased DWV-B in treated cages relative to controls. When looking within cages having natural virus levels, oxalic significantly increased DWV-B levels ($p= 0.0118$). Others have shown increases in virus levels with application of acaricides at the colony level. (Francis et al., (2013) observed an increase in DWV when honey bees were treated with organic acids or pyrethroids (synthetic acaricide). Some studies suggest that this effect could be due to the negative impact of acaracide on immunity of honey bees, leading to an elevation of virus levels (H. Boncristiani et al., 2012; Francis et al., 2013; Locke et al., 2012). Jovanovic et al. (2022), suggested increases in DWV after oxalic application could result from increases in grooming activity by honey bees, which leads to spreading of DWV among honey bees. However, this would not be likely in

our study as the impact of OA on DWV-B was not significant for orally inoculated cages which would provide little chance of exposure through grooming ($p= 0.9173$).

We orally inoculated honey bees with specific variants of DWV, to see how oxalic treatment affects caged honey bees with high levels of those viruses in the absence of varroa. The purified virus was mixed with an extraction buffer before orally feeding it to honey bees. In our second experiment and (some cages in the first experiment), equal numbers of caged honey bees were inoculated with an extraction buffer only, to provide a sham control, so that we would know if it might impact viruses. We observed the combined impacts of oxalic and buffer on DWV-A inoculated and non-inoculated cages. Although BQCV levels in oxalic treated bees decreased in comparison to no-oxalic cages. It could have been because of the combined effect of oxalic and buffer as buffer was added to DWV-A inoculated cages. This assumption was confirmed as the buffer itself reduced BQCV levels in buffer control cages in comparison to non-buffer control cages. The extraction buffer consisted of 0.1 M ascorbic acid and 0.5 M potassium phosphate. It may have contributed to viral reduction by influencing potassium ion dynamics in honey bees. Potassium plays a key role in immune regulation, particularly through K-ATP channels, which help control immune signaling and oxidative stress responses (O'Neal et al., 2017). The presence of potassium phosphate in the buffer could have modulated these channels, indirectly enhancing antiviral defenses. This mechanism aligns with findings on K-ATP channel activators, such as pinacidil, which improve viral resistance in bees (Fellows et al., 2023). Therefore, the buffer may have supported immune function by providing potassium in a way that naturally enhanced the bees' ability to combat BQCV virus. Moreover, a similar impact of the extraction buffer was observed for SBV virus. The buffer inoculated honey bees showed marginally lower SBV levels in comparison to non-inoculated honey bees.

There was a significant increase of DWV in DWV inoculated cages in comparison to non-inoculated cages for both our DWV-A and DWV-B inoculum which demonstrated our inoculation

technique was successful. However, addition of DWV-B inoculum also increased levels of other viruses. The DWV-B inoculum had 96.68% DWV-B, 3.07% DWV-A, 0.20% IAPV, 0.02% BQCV, and 0.02% SBV virus. In addition to an increase in levels of DWV-B, this inoculum also increased the levels of DWV-A, IAPV and SBV; even though they were present in trace amounts in the inoculum. It is notable that inoculum had trace levels of BQCV and SBV, but among these, only SBV levels were increased after addition of inoculum. The observed increase in SBV associated with the DWV-B inoculum could not have been due to a buffer effect as the buffer suppressed its production relative to controls in our other cage trial. When we inoculated with 99.9% pure DWV-A, as expected this pure inoculum only increased levels of DWV-A levels but had no effect on SBV. These results taken together suggest that DWV-B may have suppressed immune responses in the workers allowing SBV abundance to increase. This aligns with (Durand et al., 2024), who reported that co-infection with DWV-B and SBV synergistically enhanced SBV replication. This effect was not observed with DWV-A. Durand et al. (2024), also found that co-infection altered immune gene expression, especially by downregulating *vitellogenin* and potentially impairing antiviral responses. Similarly, Ryabov et al. (2016) showed that DWV suppresses immune pathways, including Toll and antimicrobial peptide production, which are crucial for SBV control. This may help explain why SBV, but not BQCV, increased following DWV-B inoculation despite both being present in similarly low amounts. However, it is worth noting that Desai & Currie, (2016) did not find any correlation between SBV and DWV in honey bee colonies, indicating that these interactions may be context dependent.

Temperature may also influence the efficacy of OA (Bacandritsos et al., 2007; Beyer et al., 2018; Gregorc, Knight, et al., 2017) however, was not likely a factor in this study. In the field study the temperature was 15.9 degrees C at the time of application on the day when oxalic was applied (8th Sept ember 2021) and reached a high of 22 degrees C. In the cage study oxalic was applied at room

temperature ~ 22 degrees C and then bees were held in an incubator at 32 degree Celsius. These temperatures are within the range deemed acceptable for OA effectiveness. At temperatures below 10 degree Celsius honey bees form thermoregulatory clusters, reduce their activity which potentially affects the distribution of oxalic throughout the colony (Mattila & Otis, 2007).

In addition to evaluating mite and virus dynamics, we also examined physiological responses of worker bees. Others have noted increases in lipoproteins following oxalic treatments. Sagona et al., (2024) reported immediate increase in vitellogenin levels after oxalic application, which did not persist after 48 hours. Similarly, (Pinďáková et al., 2025) showed an increase in vitellogenin levels following the application of OA trickle applications. In this study we quantified total protein in worker bees as a potential metric to act as an indicator of honey bee health. Hemolymph has the lipoprotein vitellogenin, which is major proportion of the total proteins in honey bee hemolymph. Vitellogenin plays an important role in overwintering survival of honey bees. During late fall, bees that emerge differ physiologically and these "winter bees" have higher stores of vitellogenin. Varroa feeding on adult honey bees could compromise the health of those individual bees and reduce winter longevity. In this study, total protein concentrations in worker bees were assessed on August 30, October 20, November 10, 2021, and April 27, 2022 to determine their value as predictors of colony survival since they are easily quantified. August had the highest protein levels in worker bees; October's levels declined dramatically. While still lower than in August, protein levels rose in November compared to October. Protein levels by April matched those in October and November, suggesting that protein levels stabilized during winter. Worker bees transition to winter bees in fall, so they are expected to have higher protein levels (G. V. Amdam & Omholt, 2002). Later, these proteins are used by workers to survive through winters, leading to decline in protein levels (Ward et al., 2022). We did not see any impact of OA on the level of total protein in adult

workers suggesting it may not be a sensitive enough predictor in fall for use in evaluating colony health and survival over winter.

In temperate climates as winter approaches colonies shift to the production of long lived “winter bees” and to form clusters to maintain temperature within the colony throughout the winter. In this study cluster size and colony weight were assessed prior to wintering (November 17, 2021) and following retrieval from winter storage (April 27, 2022) as indicators of colony health. Neither early oxalic treatment nor initial mite levels had a significant influence on the cluster size of colonies prior to winter or on the size of surviving colonies. This is in contrast to results of (Parsons, 2017) where early oxalic treatment resulted in larger fall cluster sizes than later oxalic treatments and greater survival of marked age cohorts of bees when oxalic was applied at the beginning of September than on later dates. Other studies also did not find oxalic treatment had any positive or negative effect on cluster size (Al Toufailia et al., 2015; Bahreini & Currie, 2015a; Jack et al., 2021; Plamondon et al., 2024; Prouty et al., 2023; Tellarini Prieto et al., 2024). Seasonal variation in honey bee cluster size in this study, showed cluster sizes were marginally smaller relative to the fall as is typical due to stress caused by varroa, pathogens and normal winter mortality (Currie & Gatien, 2006; Desai & Currie, 2016) but were not affected by OA treatment. Multiple OA acid trickle applications can cause brood mortality leading to reduced cluster size in April (Adjlane et al., 2016) however, only one application of OA was applied in this study.

In this study the levels of varroa and DWV increased from early to late fall, and were fairly high before going into the indoor wintering environment despite the early fall oxalic treatment. There were very high levels of colony death over winter and only 15% of treated colonies and 10% of untreated colonies survived survival until spring. The year in which this study was conducted in the Province of Manitoba, beekeepers had very high annual losses across the Province in that year (CAPA, 2022). Beekeepers reported losing 57% of colonies across the entire Province and survey results from

those losing greater than 25% identified ineffective varroa control, weather, “don’t know” and weak colonies in the fall as the four top responses (in that order). Colony weight loss over winter is correlated with colony size as larger clusters consume more food stores but can also be indicative of stress if bees within those colonies have higher rates of food consumption. We saw no indication that colony weight loss was affected by treatment in this study. Although the early fall application of oxalic did not show a direct effect as a predictor significantly influencing survival in this study, colonies treated in early fall did have reduced levels of both DWV-A and SBV which can be correlated with colony loss (Desai & Currie, 2016a). The number of mites a colony had in September affected virus levels later on in this study. Surprisingly, colonies that started with more mites (0.48% to 9%) had lower levels of DWV-A in the spring. Since varroa mites spread this virus, (Beaurepaire et al., 2020) this seems unexpected. However, logistic regression analysis of overwintering survival helps explain this trend. Among all the factors tested such as Nosema, cluster size, protein, and multiple viruses; only DWV-A and varroa mite levels in the fall significantly predicted colony survival. Colonies with higher DWV-A titres and greater mite infestations were less likely to survive the winter. PCA analysis suggested that predicted survival times were positively associated with oxalic treatment and BQCV but negatively associated with fall mite levels and DWV-A. This suggests that the reduction in DWV-A observed in spring was not solely due to the effects of mite levels, but rather because colonies with highest DWV-A (and possibly DWV-B) and high mite burdens were more likely to die over winter, leaving behind only the healthier, less infected ones. This aligns with findings by (Highfield et al., 2009), and similar studies by (Currie & Gatien, 2006) and (Van Dooremalen et al., 2012), who showed that high fall mite levels significantly increase winter mortality or lead to colonies with smaller populations in spring. The results of this study suggest that if levels of DWV-A are near zero colony survival of over 80% can be expected with late fall varroa levels below 10 % in agreement with late fall thresholds (Currie 2008) but if DWV-A is present in combination with varroa survival is drastically reduced. If varroa is absent DWV-A needed to be held below 10^7

genecopies to obtain 80% or higher survival. These results are in agreement with other studies that show varroa mites and DWV are significant threats to honey bee colonies (Kevill et al., 2019; Paxton et al., 2022; Plamondon et al., 2024).

In contrast to DWV-A, colonies with high initial mite levels showed high levels of BQCV compared to those with low initial mite levels. This was supported by a strong statistical effect of the initial mite block on BQCV ($F = 160.30$, $p = 0.0062$). Although, this relationship could suggest that high mite burdens may contribute to increased BQCV abundance, aligning with previous findings that BQCV can replicate in varroa mites (Sabahi et al., 2020) the ramifications for colony survival are unclear as our PCA analyses showed a positive association for BQCV and colony survival time.

Conclusion

According to previous research in our lab (Parsons, 2017), early fall application of 3.5% oxalic improved the survival of honey bees without having a significant impact on varroa levels possiblythrough suppressing viruses or improving the relative health during the formation of winter bees. In this study, I investigated whether an early fall OA trickle treatment, applied while brood was still present, could provide added protection to honey bees, either by suppressing viruses or increasing total protein levels in wintering bees beyond its known effect on varroa control.

In the field study, OA increased daily mite mortality two weeks after treatment, but resulted in no significant effect on the overall mean abundance of varroa mites over the long term. Oxalic also had no effect on cluster size, colony weight, or total protein levels in wintering bees. However, we did observe a longer term effect of the fall treatment in colonies the following spring. Colonies that received the oxalic treatment and survived the winter showed reduced DWV-A levels compared to controls, suggesting a possible delayed antiviral effect. Although daily mite fall data confirmed that OA initiated some level of

mite mortality shortly after treatment, its inability to reduce overall mite infestation levels reinforces the need to consider brood status when planning treatment. This also highlights the difference between short-term measures of treatment impact, such as mite drop, and longer-term metrics like infestation levels or overwintering survival.

In cage experiments, where bees were kept varroa-free to assess any direct impact of OA on viruses, results were inconsistent and seemed to vary depending on the virus. We were able to successfully inoculate bees with both DWV-A and DWV-B for these experiments. In the first experiment, OA increased DWV-B levels in cages with naturally infected bees, which may suggest a stress response rather than a suppressive effect. In the second experiment, OA reduced BQCV levels, but this result was influenced by the buffer used in the treatment. The buffer alone reduced both BQCV and SBV levels, which makes it difficult to isolate the effect of OA.

Overall, OA treatment in early fall may have some virus suppressing effects especially against DWV-A in the longer term but the results of this study indicated it did not improve protein levels or colony performance. In the absence of varroa, OA's direct effects on viruses are variable and require further investigation to better understand the underlying mechanisms and their practical relevance.

More studies need to be conducted to analyze the effect of oxalic on the level of viruses particularly combinations of early oxalic treatment with later follow up treatments with a variety of acaricides. The effects of early fall OA treatments are still uncertain, and more research is needed to clarify its potential as part of an integrated approach to honey bee pathogens.

Figures

Figure 2. 1: Cage setup showing the net screen inside the cage to help bees crawl to the falcon tubes. One of the tubes is supplying water, and the other one has sugar syrup and the virus inoculum.

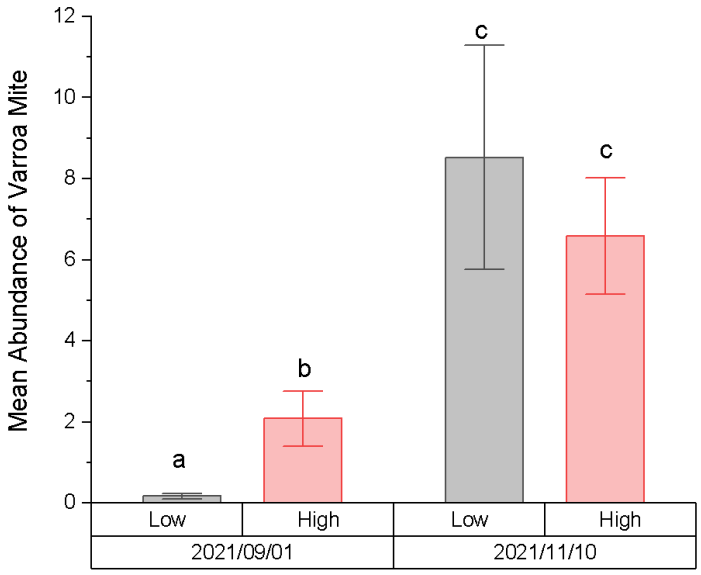

Figure 2. 2:Effect of the date and initial varroa mite level on mean abundance of varroa mites. The y-axis represents the mean abundance of varroa mites and the standard error. The x-axis represents the dates (2021/09/01 and 2021/11/10) on which samples were collected. The initial mite block (Low or High) was determined by the mean abundance of varroa mites prior to assignment to treatments on 2021/09/01. The means followed by the same letter are not significantly different from each other (Bonferroni P > 0.05).

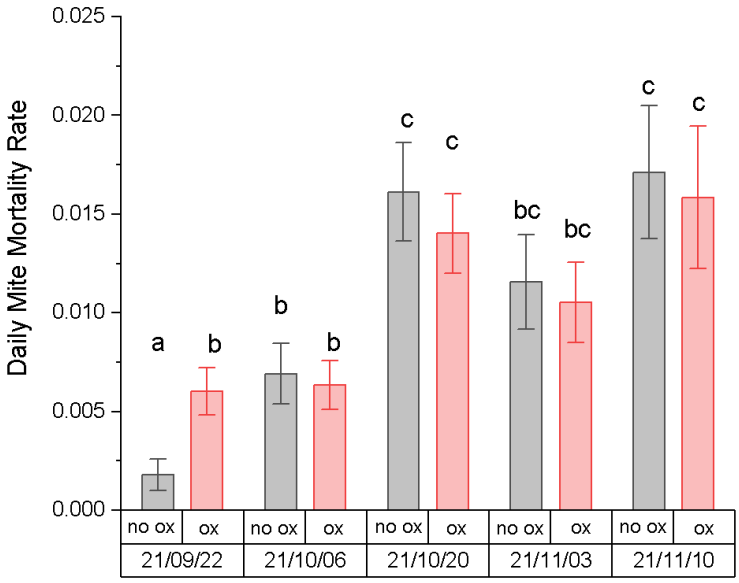

Figure 2. 3: The effect of the interaction of date and oxalic treatment on daily mite mortality rate (DMMR). The y-axis represents DMMR which is the proportion of mites in the colony falling from onto sticky boards in the specific period. The x-axis represents the date sticky boards were pulled out and the treatment (No Oxalic and Oxalic). OA trickle was applied on 2021/09/08. The bars followed by the same letter are not significantly different from each other (Bonferroni P > 0.05).

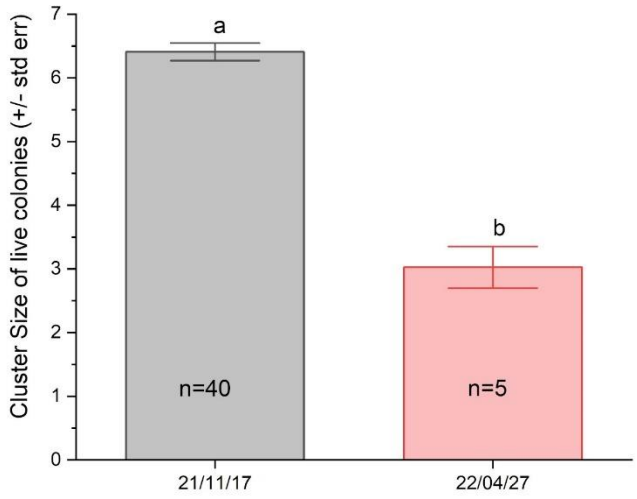

Figure 2. 4: Variation in cluster size of live colonies before and after wintering indoors. The y-axis represents the cluster size of colonies and the standard error, excluding dead colonies with zero cluster sizes (n=number of live colonies). The x-axis represents the dates (2021/11/17 and 2022/04/27) on which the cluster size was recorded. The bars followed with the same letters are not significantly different from each other (Bonferroni $P > 0.05$).

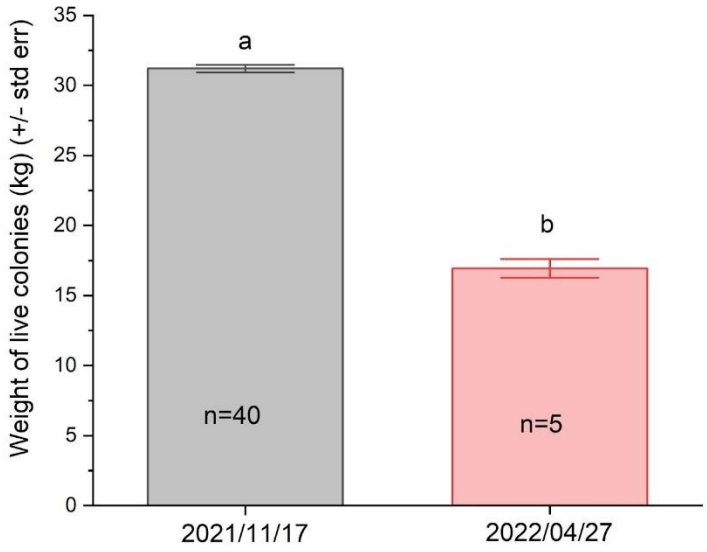

Figure 2. 5: Variation in colony weights of live colonies before and after wintering indoors. The y-axis represents the weight of colonies and the standard error, excluding dead colonies with zero cluster sizes (n=number of live colonies). The x-axis represents the dates (2021/11/17 and 2022/04/27) on which the colony weight was recorded. The bars followed with the same letters are not significantly different from each other (Bonferroni $P > 0.05$).

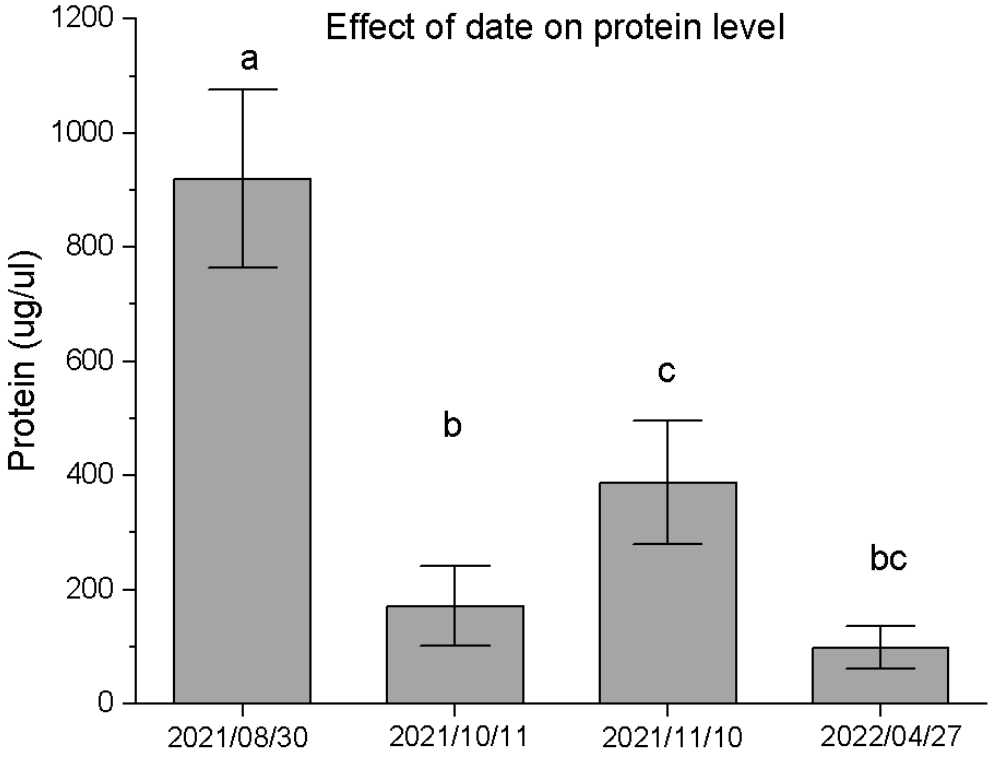

Figure 2. 6: Effect of early fall varroa abundance on total protein level (+/- standard error) (µg/µl) in workers from early fall to the following spring. Data are presented by date. Bars followed by the same lowercase letter are not significantly different from each other (Bonferroni P > 0.05).

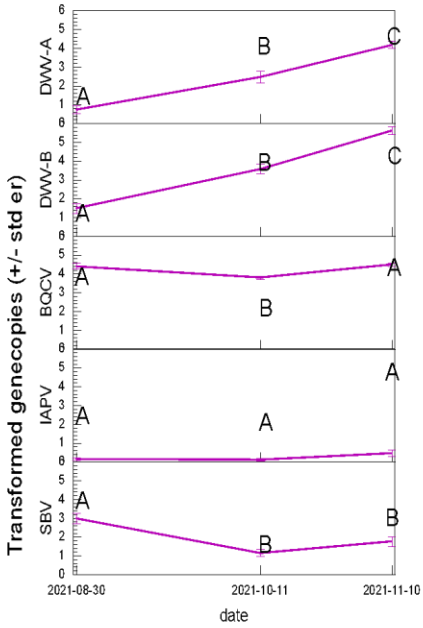

Figure 2. 7: Variation in virus levels for (DWV-A, DWV-B, BQCV, IAPV, and SBV) across dates in the fall of 2023. The y-axis represents log-transformed viral gene copies and the standard error. The x-axis represents the dates (2021/08/30, 2021/10/11, and 2021/11/10) on which the samples were collected. In each panel, the points with the same letters are not significantly different from each other (Bonferroni P > 0.05).

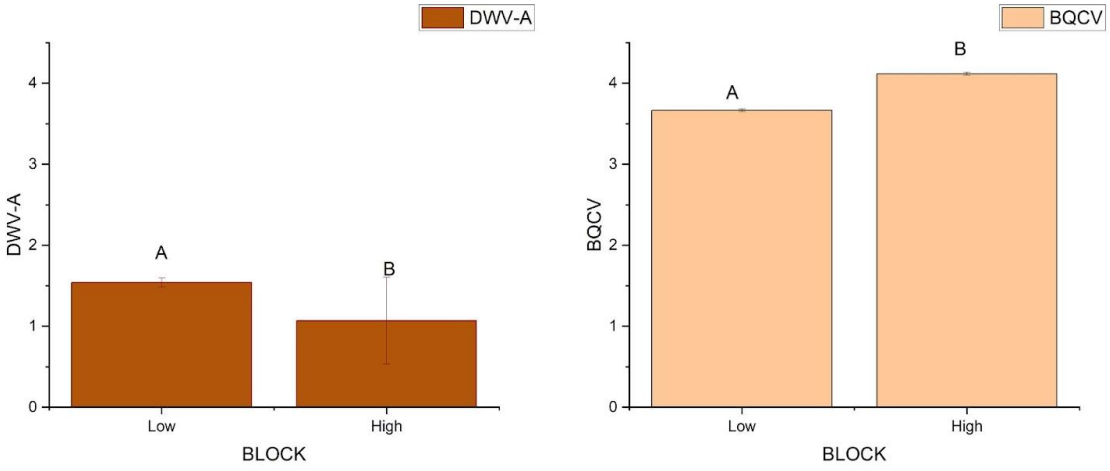

Figure 2. 8: Variation in virus levels (DWV-A and BQCV) in April for different initial varroa mite blocks. The y-axis represents log-transformed viral gene copies of surviving colonies and the standard error. The x-axis represents the initial varroa mite block (Low and High) which was determined by the mean abundance of varroa mites on 2021/09/01. In each panel, the bars followed by the same letter are not significantly different from each other (Bonferroni P > 0.05).

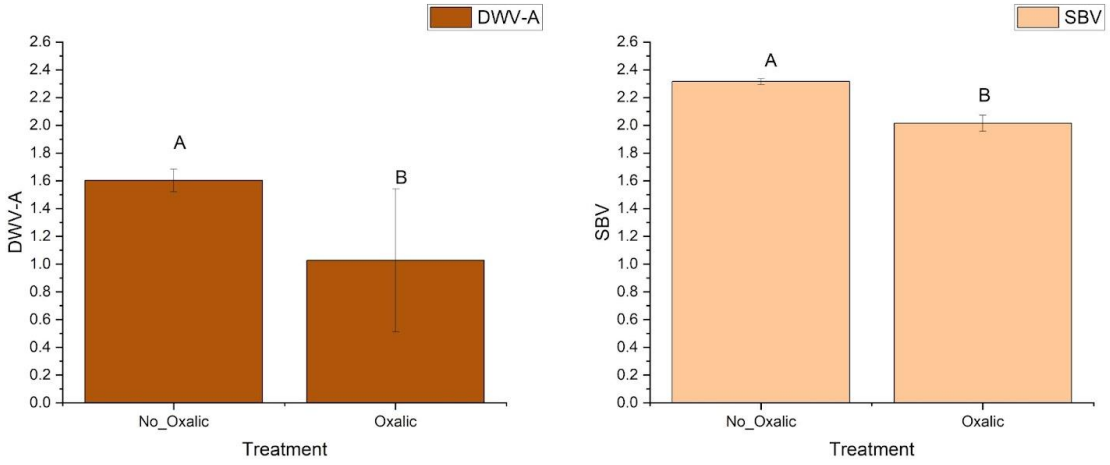

Figure 2. 9: Variation in virus levels (DWV-A and SBV) in April for different oxalic acid treatments (No oxalic and oxalic). The y-axis represents log-transformed viral gene copies of surviving colonies and the standard error. The x-axis represents treatment (No oxalic and oxalic) which was applied on 2021/09/08. In each panel, the bars points followed by the same letter within viruses are not significantly different from each other (Bonferroni P > 0.05).

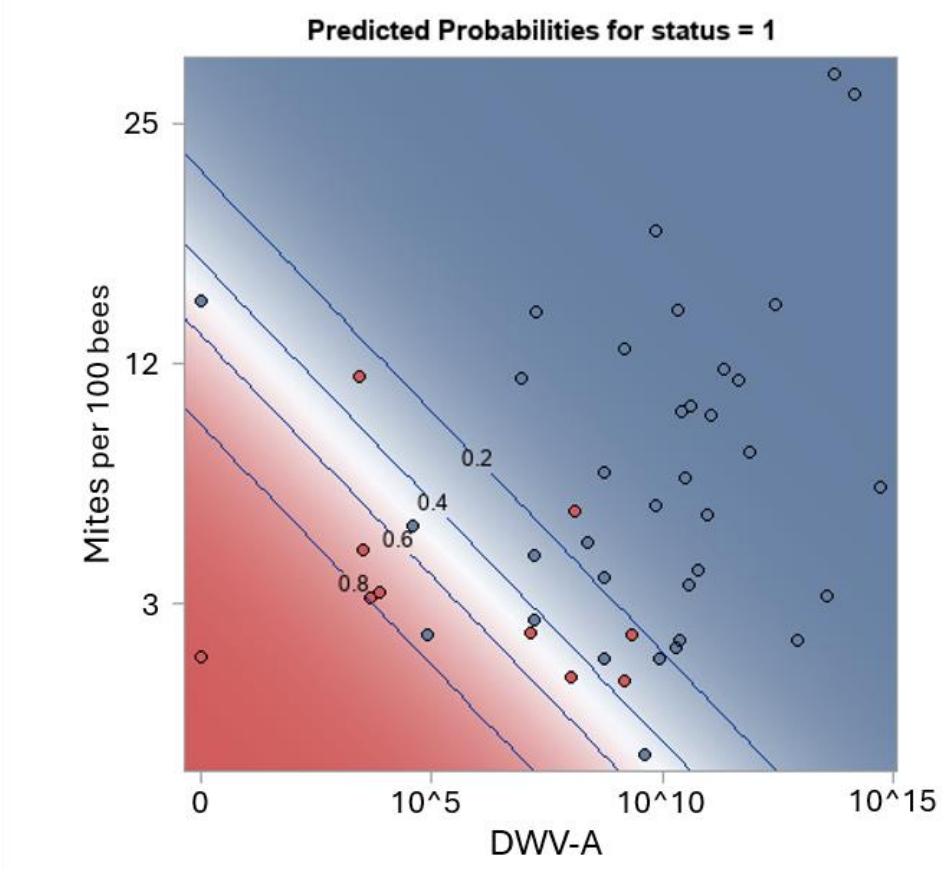

Figure 2. 10: Fall predictors of colony mortality in spring. The analysis evaluated the effects of early fall oxalic treatment, late fall virus levels (DWV-A, DWV-B, BQCV, IAPV, SBV), nosema, varroa infestation, and colony cluster size and colony weight on overwintering survival using November data. After backwards selection, DWV-A gene copies per µl RNA and varroa mean abundance (mites per 100 bees) were retained as significant predictors in the final logistic regression model. Diagonal lines indicate the probablity of colony survival given combinations of varroa mite abundance (Mites per 100 bees) and DWV-A gene copies. The graph displays untransformed values for interpretability.

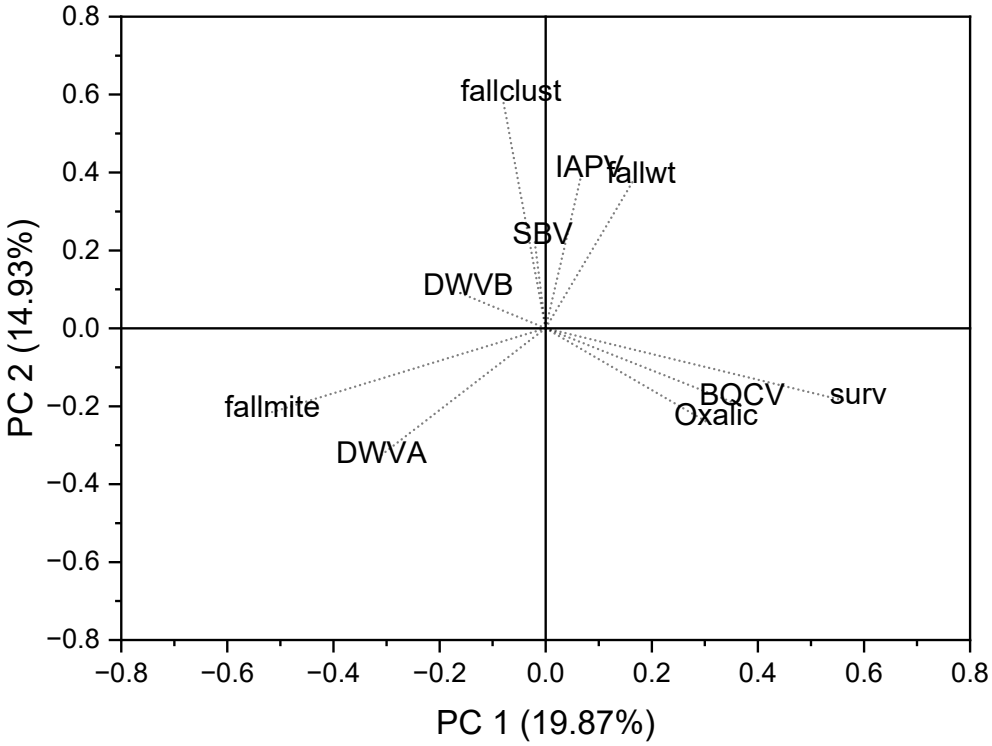

Figure 2. 11:Principal Component Analysis (PCA) loading plot showing the relationships among oxalic acid treatment, fall mite levels, overwinter survival, fall weight, and virus infections. Variables are represented as vectors based on their loadings. The alignment of survival, oxalic and BQCV vectors indicates a positive association, while fallmite and DWVA vectors point in the opposite direction, suggesting negative correlations with survival.

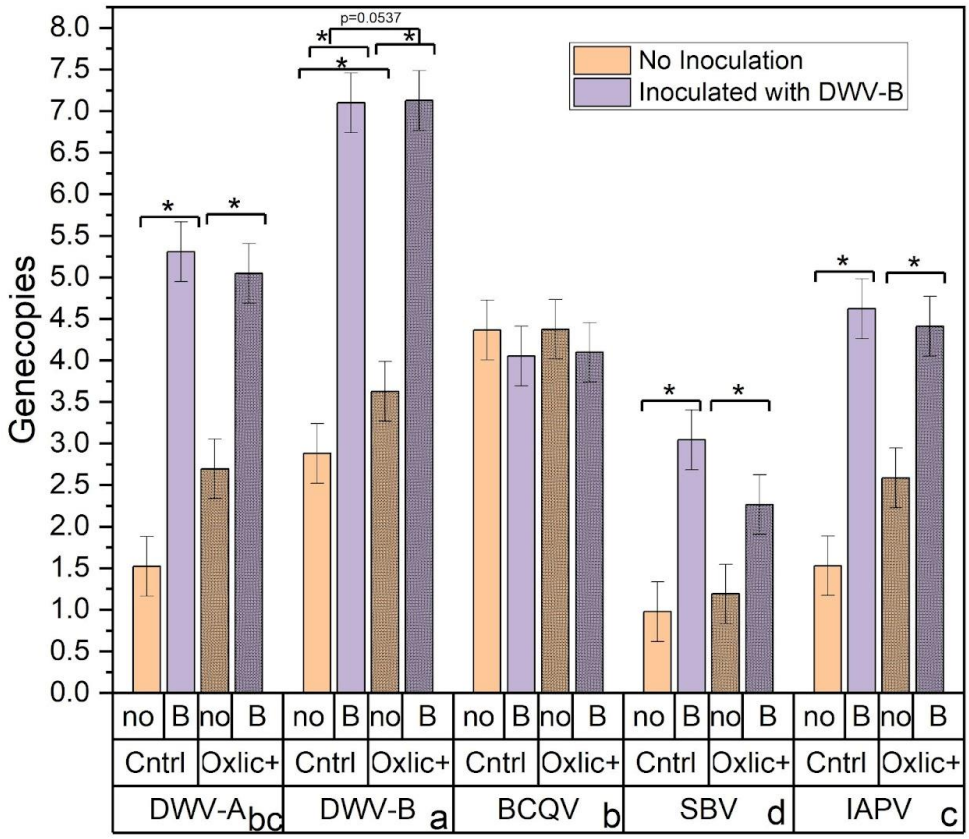

Figure 2. 12: Variation in different virus types across cages with different treatment regimes. The y-axis represents transformed gene copies and the standard error, while the x-axis displays virus types (DWV-A, DWV-B, BQCV, SBV, and IAPV). Oxalic+=Oxalic acid treatment groups, and Cntrl=control colonies. No=Non-inoculated cages (orange) and B=DWV-B inoculated cages (purple) are distinguished. Bars marked with an asterisk (*) indicate significant differences, while portions labeled with different letters are also significantly different from each other.

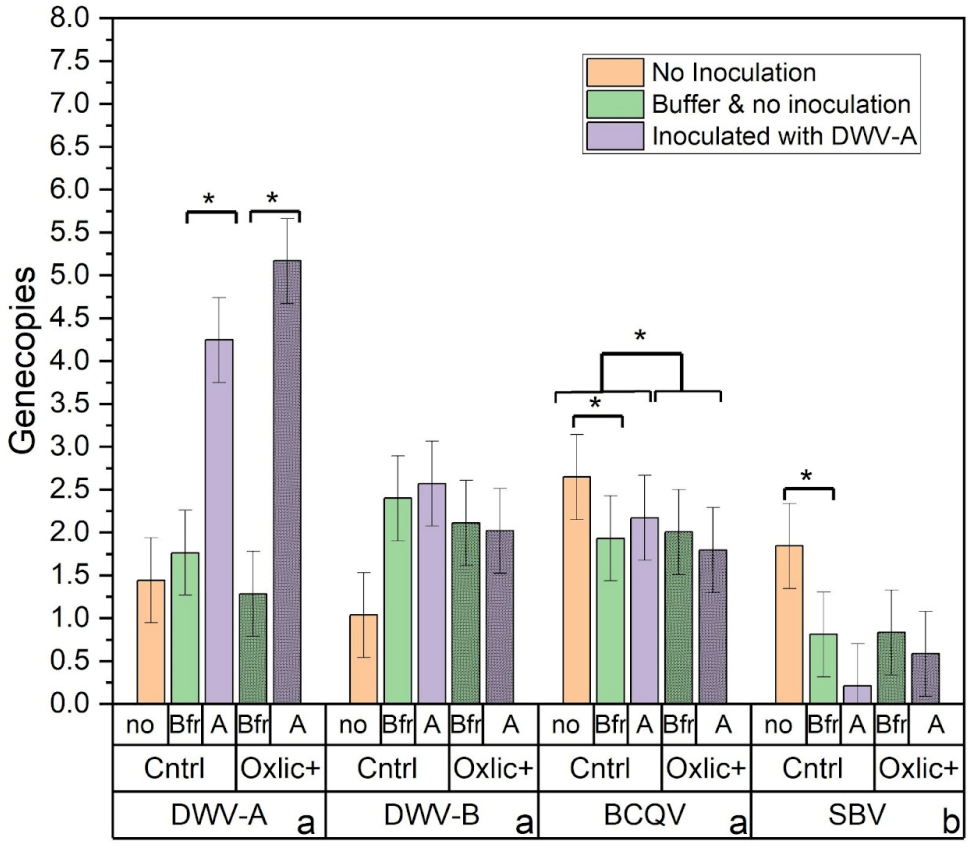

Figure 2. 13**:** Variation in different virus types across cages with different treatment regimes. The y-axis represents transformed gene copies and the standard error, while the x-axis displays virus types (DWV-A, DWV-B, BQCV, SBV). Oxalic+=Oxalic acid treatment groups, and Cntrl= control colonies. No=Non-inoculated cages (orange), Bfr=buffer-inoculated cages (green), and A=DWV-A inoculated cages (purple) are distinguished. Bars marked with an asterisk (*) indicate significant differences, while portions labeled with different letters are also significantly different from each other.

Chapter 3: Effect of Number and Interval of Fall Oxalic Treatments under Two Wintering Environments on Efficacy Against Varroa Mites, Pathogens and Winter Survival of Honey Bee Colonies

Abstract

Honey bee colonies suffer high rates of annual loss that are attributed to varroa and its interaction with viruses and other stressors. This study assessed the number of applications of oxalic acid (OA) vapour treatments needed for varroa mite control, the most effective timing for multiple doses and their impacts on colony health. Varroa infested colonies were first treated in late fall in a North temperate climate with OA vapour using zero, one or four applications. Second, we compared four applications of OA at intervals concentrated at the end of the fall period (4 to 5 days) or spread out throughout the fall (12 days between treatments) and compared these to untreated colonies. In each experiment, half of the colonies from each treatment group were wintered indoors, and half were wintered outdoors. Colony varroa populations were sampled using sticky boards and alcohol wash and colonies were assessed to determine cluster size, colony weight, total protein and viral load of workers. Although sticky boards indicated both one and four OA treatments caused mite mortality, varroa levels were reduced relative to the control in spring only by four applications of OA. Colonies treated four times had greater cluster sizes in fall and lower mite levels in spring but cluster size or number of surviving colonies was not improved. Treatments at 12 or 5-day intervals both affected varroa mortality in winter but did not reduce the mean

abundance of mites in fall or spring. Later treatment at 5 day intervals increased colony survival in spring relative to controls but only when initial fall mite levels were very low. Worker total protein and virus levels were not directly affected by OA treatment. Colonies with low initial mite levels had lower DWV-B levels than those with high initial mite levels but only when treated with OA. Colonies wintered indoors had lower DWV levels than colonies wintered outdoors in both years.

Key Words: Oxalic acid, Colony health, Varroa efficacy, Fall treatment, Deformed Wing Virus (DWV)

Introduction

Honey bees are essential pollinators in agroecosystems, supporting the pollination of about one-third of crops worldwide (Delaplane & Mayer, 2000; Klein et al., 2007; McGregor, 1976). They are presently threatened by parasites, viruses, diseases and their interactions with other stressors such as lack of nutrition, pesticides and climate (Flores et al., 2021). High rates of annual colony loss jeopardize sustainable agroecosystems by hindering pollination services (Watanabe, 1994). Much of this loss is attributed to the varroa mite, *Varroa destructor* (Anderson and Trueman), an ectoparasite that if not controlled causes considerable economic loss (Rosenkranz et al., 2010). Therefore, it is crucial to manage economic losses caused by varroa to achieve sustainable food production and maintain production of honey and other products produced by these bees (Baylis et al., 2021).

Varroa has two stages in its lifecycle - reproductive and “phoretic” stages (Wilkinson et al., 2001). Varroa reproduces inside honey bee brood cells where both bee and mite development occur within protected bee cells covered with a capping (Garrido & Rosenkranz, 2003). When the mite is under the protective capping of the brood cell it is also protected from exposure to many acaricides such as OA that

work through direct contact. During the phoretic stage, the varroa mites feed on and transfer from one adult honey bee to another (Salamanca Grosso et al., 2012) and the mite is more exposed to potential contact treatments. One female mite can go through up to three reproductive phases during their lifetime (Nazzi & Le Conte, 2016). Brood availablity that allows mites to reproduce within colonies varies seasonally. In North temperate climates, the queen bee reduces egg-laying in late summer, fall and during winter so most mites are in the phoretic phase. Although some brood rearing occurs in mid-winter its utilization by varroa for successful reproduction is very low (Kozak, 2008). During this "broodless phase", most of the mite population is susceptible to contact acaricides.

Varroa is highly destructive to both the adult and immature (brood) stages of honey bees, *Apis mellifera L. (Traynor et al., 2020)*. When feeding, varroa punctures the cuticle of a honey bee making it more susceptible to bacterial and viral infections (Kanbar & Engels, 2003). It also feeds on the haemolymph and fat body of honey bees and acts as a vector for viruses like deformed wing virus (DWV) (Annoscia et al., 2019; Han et al., 2024; Ramsey et al., 2019). Due to feeding by *Varroa,* the weight of emerging bees is reduced and reductions in haemolymph protein content occur relative to non-infested honey bees (Bowen-Walker et al., 1999; Bowen-Walker & Gunn, 2001; Dandeu et al., 1991; Van Dooremalen et al., 2013) in addition to compromising the bee's immune system making it susceptible to other pathogens (Morfin et al., 2023). Economic impacts of the mite are severe and honey bee colonies can collapse within two years if not treated for varroa (Le Conte et al., 2010). Thus, control of varroa is a major concern for beekeepers every year (Steinhauer et al., 2023). Substantial resources are devoted to research to provide beekeepers with effective and immediate solutions to control varroa mites (Bubnič et al., 2021). Although many acaricides have been used to protect colonies from varroa mites, their efficacy is often reduced due to the mites developing resistance against these chemicals (Adjlane et al., 2012). For example, synthetic pyrethroid acaricides (Apistan ®, Bayvarol ®) and the organophosphate coumaphos

(CheckMite+) were registered for use as chemical controls, but due to the devlopment of resistance to them, they are not consistently effective in much of the world including Canada (Adjlane et al., 2013; Currie et al., 2010). Other compounds like formulations of amitraz (Apivar®) are currently used (Jack & Ellis, 2021; Maggi et al., 2009, 2011; Rinkevich, 2020; Sammataro et al., 2005) but have been documented to be resistant to mite (Elzen et al., 2000). Therefore, it is becoming important to provide beekeepers with new solutions to mitigate the impacts of varroa mites. Much research has been focused on organic acids like OA and formic acid, that are attractive alternatives as they are natural components of honey (Bogdanov et al., 2002) and less likely to cause problems with residues in honey. Beginning in the 1980s, OA was developed as natural product that is considered an alternative miticide to treat varroa (Okada & Nakane, 1987; Rinkevich, 2020) and it was first registered for use in North America (Canada) in 2010 (PMRA, n.d.). Resistance to OA for control of varroa has not yet been observed (Berry et al., 2022; Maggi et al., 2017). It is also a sustainable solution as it has minimal human risk quotient, rarely contaminates bee products and is a component of many food items including honey (Kirkland et al., 2005; Rademacher & Harz, 2006). In Europe and Asia, multiple delivery methods, such as spraying, trickling, and sublimation are used to apply this compound (Nanetti et al., 2003). Applications to colonies by trickling and spraying methods use OA mixed in sugar syrup (Charrière et al., 1998; Imdorf, Charrière, et al., 1995; Nanetti & Stradi, 1997) applied directly onto the colony's workers. In contrast, the sublimation method uses a device to rapidly heat OA crystals and fumigate the colony (Radetzki, 2001). Applying OA by sublimation is considered by some to be safer for honey bees as it allows using smaller doses of active ingredient per colony than spraying or trickling (Al Toufailia et al., 2015).

Oxalic is most effective when the brood is absent because it has little to no efficacy against reproducing mites under the capped brood cells (Gregorc et al., 2017). However, during a broodless periods, all of the varroa mites have potential to be exposed to treatment, potentially making applications

more efficacious (Rademacher & Harz, 2006). In North temperate regions, a broodless phase typically occurs at some point in late fall or throughout the winter as the colony ceases brood rearing and stores resources for winter survival (L. Harris, 2017) although some brood is present in most colonies at some point in winter. Honey bee worker cells are capped for 12 days as they develop from prepupae to adults. Therefore, applying multiple OA applications at 12-day intervals throughout the fall might better expose mites emerging during the fall period to the treatment during periods of favourable temperatures while helping to suppress the impacts of varroa-related stress and its associated pathogens. In contrast, concentrating multiple treatments late in the fall when little to no brood is present might increase efficacy against the mites although it would potentially occur when climatic conditions are more variable. Treatments against varroa in the fall used to bring mite levels down are particularly important as high mite levels in fall have a major impact on colony survival (Currie & Gatien, 2006; Delaplane et al., 2005; Guzman-Novoa et al., 2016; Morfin et al., 2024; Van Dooremalen et al., 2012).

The primary objectives of this project were to first to assess what number of applications would be required for effective control using the vaporization method in late fall by comparing one, four or no treatments. Second, was to evaluate the efficacy of multiple (four) OA applications at two intervals in fall (starting in early fall and continuing every 12 days and starting in late fall and continuing every 4-5 days) against varroa. In the later experiment a total of four OA treatments were applied during late fall at different intervals and compared to untreated colonies. Third, we evaluated the effects of these treatments under two wintering management methods (indoor and outdoor) with different initial mite loads. We also assessed the impact of the different OA treatment strategies on honey bee colony health and survival, which was measured by analyzing total protein levels, viral loads in the bees, colony cluster size, colony weight and colony survival.

Materials and Methods

Experiment 1: Effect of late fall oxalic acid treatment frequency and wintering method on efficacy and colony performance

Experiment one was designed to assess the efficacy and impact of varying the number of late fall OA vapour treatments on varroa abundance and colony performance in different wintering environments. The experiment was performed using 60 single brood chamber colonies maintained at the Honey House yard on the Fort Garry Campus of the University of Manitoba (49°48'31"N 97°07'37"W). Colonies were assessed to quantify the mean abundance of varroa mites on 27 October, 2020 and colonies were assessed to quantify pretreatment cluster scores on 12 November. To ensure a relatively equal distribution of mites among treatment groups colonies were first grouped into 10 mite range blocks based on initial mite level with 6 colonies in each block ((1= 0.8 to 1.1; 2 = 1.1 to 1.3; 3 = 1.3 to 1.6; 4 = 1.6 to 1.9; 5 = 2.1 to 2.7; 6 = 2.8 to 4.3; 7 = 4.4 to 5.3; 8 = 6.3 to 9.3; 9 = 9.4 to 12.3; 10 = 18 to 66; mites per 100 bees) and randomly assigned to three treatments groups: an untreated control, one application of 1 gram of OA on 13 Nov. and 4 applications of 1 gram of OA on 13, 16, 18 and 23 Nov. spaced 2 to 5 days apart within each wintering method. Fresh sticky boards were placed under each colony in the experiment at the time of each treatment and replaced throughout the winter on a regular basis (13 to 16 Nov (3 days); 16-19 Nov, (3 days); 19-23 Nov (4 days); 23-26 Nov (3 days), 26 Nov to 3 Dec. (7 days); 3-17 Dec (14 days), and 17 Dec to 3 Feb (48 days), 3 Feb-5 Mar.(30 days), 5-26 Mar. (21 days), 26 March to 2 April (7 days) with the final board being removed on 10 April 2021 (8 days).

Samples of approximately 300 to 350 bees were collected from each colony on 26 November 2020 and 1 April 2021 to assess the mean abundance of varroa, measure total protein and viruses (protein and viruses were only quantified in surviving spring colonies in this experiment). Colonies were also

weighed and cluster scores were measured on 26 November, 2020 prior to assignment to wintering treatments and in the spring on 1 April 2021. Colonies were fed *ad libitum* throughout the fall with 2:1 sucrose syrup but any colonies weighing less than 60 pounds in late November were provided 2.5 additional feed frames and colonies between 60 and 70 pounds received an additional 1.5 frames of feed. After feeding colonies were reweighed on 27 Nov. before moving them to their respective wintering environments. On 30 November, half the colonies in each treatment group were randomly assigned to be wintered outdoors and half wintered indoors using standard management for the region.

Experiment 2: Effect of timing and interval of fall oxalic acid treatments on efficacy and colony performance

The second experiment was performed using 60 single brood chamber colonies maintained at the Observatory yard, Glenlea research station, Manitoba (49°38'44"N 97°07'21"W). Colonies were sampled to assess the mean abundance of varroa mites before the experiment (2021/09/22). Based on the relative mite abundance, the colonies were divided into two blocks: “low” mite level (0-0.7 mites per 100 bees) and “high” mite (0.6-13.7 mites per 100 bees) abundance and then randomly assigned to treatments within each block by using a random number generator from Excel. For each mite block, ten colonies were treated in early fall – four times with OA vapour at an interval of 12 days (2021-Sept-27, 2021-Oct-09, 2021-Oct-21, 2021-Nov-02), an additional ten colonies were treated four times in late fall at an interval of 5 days (2021-Oct-19, 2021-Oct-24, 2021-Oct-29, 2021-Nov-03), and ten colonies were untreated control colonies. OA was applied using the sublimation/vapor method with a dose of 1 g OA applied according to label recommendations for the Province of Manitoba and for the, ProVap, applicator to single brood chamber colonies. The OA was placed in the device and heated to dissipate the acid until all was gone (approximately 20 seconds per hive). Colony entrances were sealed during active treatment

as per label recommendations. Following the final treatments, which occurred on the same day for both OA treatment regimes, half of the '5-day interval' treatment colonies, half of the '12-day interval' treatment colonies, and half of the untreated colonies were randomly selected to be moved to an overwintering building (described in chapter 2), and the remaining half were placed outdoors in the honey house yard on campus at the University of Manitoba. The outdoor wintered colonies were covered with fiberglass insulating material (minimum of R12 on the sides and R22 on top) and wrapped in plastic according to standard practice for the region (Gruszka, 1998). All colonies were provided with an inner cover and a top entrance. The indoor wintered colonies were fed during the experiment as needed, as described in Chapter 2. As outdoor colonies could not accommodate entrance feeders in mid-winter due to cold, they were fed twice with frame feeders in February to mitigate any effects of starvation on colony survival.

Data collection

Colonies were sampled to collect worker bees from the central brood area of colonies. For analyses of total protein (30 bees), and virus levels (30 bees) and to assess the mean abundance of varroa (~300 bees) before the OA treatments were applied (2021-Sept-22), before overwintering (2021-Nov-08) and then in spring (2022-Apr-27). Bees sampled for total protein and virus quantification were placed on ice immediately after collection and transported to -80° celsius freezer. Cluster sizes of colonies were recorded before winter (2021-Nov-17), and in spring (2022-Apr-27) by examining hives to count the number of frames covered by bees as observed from above and below each brood chamber as described in chapter 2 (Chabert et al., 2021).

The number of varroa mites falling from the colonies was also monitored continuously throughout the study using sticky boards (as described above chapter 2) that were placed under colonies

on the first treatment date and then replaced on subsequent treatment dates so daily mite mortality rates (DMMR) could be quantified. Out of the 20 untreated control colonies in the fall period, the sticky boards for half of the colonies were replaced at the same time as the '12-day interval' (27 Sept. to 09 Oct.; 09 to 21 Oct.; 21 Oct. to 02 Nov.; 02 to 09 Nov. (7 days)) treatments, and the remaining half were replaced at the same time as the '5-day interval' ((19- 24 Oct., 24-29 Oct., 29 Oct. to 03 Nov., 03 to 09 Nov. (6 days)) treatment dates. Additionally, all of the sticky boards were changed before and after moving the bees from Glenlea research station to the campus (9 to 12 Nov., 2021) in preparation for wintering (12 to 19 Nov.) and after moving the bees into the wintering building (19 Nov.). During the winter, sticky boards were replaced at least once every month for all treatment groups (19 Nov. to 20 Dec., 20 Dec. to 20 Jan., 20 Jan. to 22 Feb., 22 Feb. to 18 Mar., 18 Mar. to 22 April). Any colonies recorded as live in spring (with a queen and live worker bees) were fitted with sticky boards and treated after the last sample collection of workers with Apivar strips (42 days long treatment 2022-Apr-28 to 2022-May-24) to quantify the total numbers of mites remaining in the colonies. This was done so that the total numbers of mites in the hive at the start of the study could be determined for use in calculating DMMRs. At the time of the experiment no cases of Amitraz resistance occurred within the Province of Manitoba so it is assumed that most of the remaining mites in the colony were killed by the Amitraz treatment.

Protein and Viral Analyses

Honey bee samples were at -80º C immediately after collection to assess virus levels and total protein. Bees were transferred from the freezer to the lab in liquid nitrogen prior to homogenization. Thirty bees from each colony sample were selected, transferred into 15 ml cryotubes along with two 9 mm beads and shaken at a speed of 1750 rpm for 3 minutes in Spex™ SamplePrep Geno/Grinder 2010. A volume of 0.1 ml from the homogenate was placed into tubes (1.5ml) for analysis of virus and protein,

while the remainder was saved and stored at -80°C. Each sample was weighed. Samples were stored on ice throughout the procedure while handling.

For analyses of virus, the homogenized samples were then analyzed with appropriate gBlocks as standards (Appendix 1) and primers (Appendix 2) to quantify DWV-A, DWV-B, BQCV, IAPV and SBV levels. Extraction of RNA for viral analyses was done using the Qiagen RNeasy Mini kit. Duplicate samples from each colony were used to determine the concentration of RNA (ng/µl) and the absorbance (260/280) using a Nanodrop™ 8000 Spectrophotometer. This RNA was stored in a -80°C freezer for further analysis. An iScript™ cDNA Synthesis kit was used for cDNA synthesis using 2 µg of RNA. The cDNA was stored in a -20°C freezer before further analysis. The sample was diluted with 20 µl Molecular Grade Water (MGW) or as much needed to obtain a 2:1 dilution.

For quantification, each well in the PCR plate received 10µl Eva Green supermix (Bio-Rad™), 1µl of primer, 7µl of MGW, 2 µl of cDNA or G block. Samples were run in triplicates, each triplicate had one actin control. Samples were analysed for DWV-A, DWV-B, BQCV, IAPV and SBV. On each virus plate, absolute quantification of gene copies was obtained by running standard curves with G block gene fragments (Integrated DNA Technologies™). Concentrations ranging from 10^4 to 10^8 were utilized to establish the standard curves. The plate was placed in a CFX96™ Real-Time System (Bio-Rad™) for processing. The qPCR was performed with an initial denaturation step at 95°C for 3 minutes, followed by 39 cycles of denaturation at 95°C for 15 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds. A plate read was conducted at the end of each cycle. After these cycles, a melt curve analysis was performed starting at 65°C for 31 seconds, followed by 65°C for 5 seconds with an increment of 0.5°C per cycle. The ramp rate was set to 0.5°C per second, with plate reads after each cycle, repeated 60 times.

Total protein concentration was determined using the Bradford assay (ThermoFisher Scientific, 2011). Buffer was prepared using Phosphate buffered saline + 0.5% Triton-X . A volume of 1,000 µl of prepared buffer was introduced to the 100 µl of homogenate. After vortexing, the specimen was kept on ice for 30 minutes and subsequently evaluated for protein concentration using a Pierce™ Bradford Protein Assay Kit (Thermo-Scientific). The standard curves for measuring protein concentration in the samples were created by utilizing bovine serum albumin (BSA**).** Absorbance at 595 of samples and standards was quantified to determine the protein concentration using a Nanodrop™ 8000 Spectrophotometer (Thermo Scientific™).

Statistical analysis

Data were analyzed using repeated measures analyses of variance where the colony (hive) was the experimental unit, and the date of sampling was a repeated measure. Data were first assessed in Origin 2023® to check for normality and homogeneity of variance. If the data violated the assumptions of the Analysis of Variance, appropriate transformations were done to improve normality and homogeneity of variance. Data were analyzed using Proc mixed (online SAS studio 2023). The main effect factors were Oxalic treatment regime, block (initial abundance (for experiment 2)), wintering method (indoor or outdoor) and their interactions. Each response variable was run under different covariance structures, and the one with the lowest AIC was selected. Where significant differences among treatments were found, post hoc tests were performed with Tukey's adjustment. In cases where significant interactions were found with date, a separate Analysis of Variance (Proc mixed) was performed for each date.

The mean abundance of varroa mites was analyzed together for fall and spring. Proportions for the mean abundance of varroa were transformed using an arcsine transformation prior to analyses. Data

for experiment 1 was analysed using an unstructured covariance structure and experiment 2 was analyzed using an “autoregressive heterogeneous (arh(1))” covariance structure.

Sticky boards were assessed by counting all of the varroa mites that fell on each board throughout the experiment. For experiment 2, the total number of mites that dropped on each board were analysed after log transformation to improve homogeneity of variance. A mixed model analysis was used to examine changes in total mite drop over time with a repeated measures analysis of OA treatment and wintering method as main effects in the model, colony as the subject and date as a repeated measure and compound symmetry as the covariance structure. Separate analyses were applied on dates prior to and following assignment of colonies to different wintering treatments. Where significant treatment*date interactions occurred separate analyses by date were done with treatment means being compared after Tukey-Kramer adjustment. For experiment 2, the values for mite drop on each sampling period were converted to a “DMMR” (based on the total number of mites in the colony at the beginning of the experiment) and adjusted for the number in the prior sampling period to account for variations of mite level among hives. The mortality rate for each date was calculated by dividing the value on the sticky board by the total mite count in the previous period. Finally, the DMMR was obtained by dividing the mortality rate by the number of days the sticky board was placed inside the hive. The sticky boards of half of the control colonies, which were changed at a 5-day interval, belonged to colonies from the low initial mite block. Similarly, the sticky boards of the control colonies that were changed at a 12-day interval were all from the high initial mite block. Consequently, the “DMMR” data was analyzed using a mixed model, accounting for both date and block. Least square means were compared only between treatments with their matched controls when sampling occurred on the same day. Thus, the 4-day control group was compared only to the low-mite block of the 4-day treated colonies, while the 12-day control group was compared to the high-mite block of the 12-day treated colonies for the period in the fall prior to wintering.

Sticky boards for all the colonies were changed on the same day throughout the winter from November 09, 2021. Therefore, the 4-day and 12-day control groups were treated similarly as control groups for comparison. This analysis utilized a variance component "vc" covariance structure.

The cluster size, colony weight, total protein and virus data was analyzed together for fall and spring. For experiment 1, data for cluster size not transformed but for experiment 2, was square root transformed. Data was analyzed using a compound symmetry covariance structure for experiment 1 and an "autoregressive heterogeneous arh(1)" covariance structure. For experiment 1, data were not transformed and analysed using an unstructured covariance structure, while for experiment 2, colony weights were log-transformed and analyzed using an "autoregressive heterogeneous (arh(1))" covariance structure. Data for protein was not analysed in experiment 1. In experiment 2 protein values were log-transformed prior to analyses and analyzed using a "compound symmetry (cs) covariance structure.

For experiment 1, viruses were quantified only once in the spring using a mixed model anova by virus. For experiment 2, virus data were first analyzed together for fall and spring. The virus data was log-transformed prior to analyses with different covariance structures used for each virus. The covariance structure for DWV-A was unstructured (un), for IAPV and SBV it was arh (1), and for DWV-B, and BQCV, it was variance components (vc).

For experiment 2, colony survival was analyzed using binary logistic regression (proc logistic), where colony status (alive or dead) was the response variable, and the colony (hive) was the experimental unit. Predictor variables included log-transformed virus titers (DWV-A, DWV-B, BQCV, SBV, and IAPV), log-transformed colony weight, protein content, and cluster size, as well as arcsine square root-transformed *Varroa* infestation levels. The variables treatment, *Varroa* infestation block, and Nosema

infection status were included as categorical class effects. A backward stepwise model selection procedure was used to retain only significant predictors in the final model. Odds ratios and confidence intervals were used to assess the strength and direction of associations between predictor variables and the likelihood of colony mortality. Virus data from November only were included in this analysis to reflect colony conditions at the onset of winter. All transformed predictor variables were assessed for multicollinearity prior to inclusion in the final model.

Results

Experiment 1:

Mean Abundance of varroa mites

Overall analyses of the main effects of OA treatment, and wintering method indicated that the effects of OA treatments varied with date ($F=2.94$; d.f. 6, 54; $p=0.0147$) but there were no overall effects of wintering method ($F=0.05$; d.f. 1, 54; $p=0.8252$) or interactions with wintering method and either date or treatment ($P>0.05$). Therefore, separate analyses were performed on each date. Prior to the application of OA on 12 November, colonies in all three OA treatment groups had similar levels of varroa mites groups ($F=0.00$; d.f. 2, 57; $p=0. 9959$). There was a marginal effect of OA on varroa abundance on 26-Nov-2020 ($F=3.10$; d.f. 2, 54; $p=0. 0.0533$) but the 4 late fall applications of OA significantly reduced varroa abundance relative to controls on 31-Mar-2021 ($F=7.18$; d.f. 2, 34; $p=0.0025$), and 27-Apr-2021 ($F=4.20$; d.f. 2, 30; $p=0.0246$), but it was not significantly different than single application (Figure 3.1).

Varroa mite drop

Prior to the assignment of wintering treatments the overall average for total mite drop on days when boards were removed (16, 19, 24 and 30 Nov) was affected by OA treatments (F=23.02; d.f.2, 57; p=0.001) with both 1 (65.3 $\pm$ 28.2) application and 4 applications (122.7 $\pm$ 28.1) of OA having greater average total mite drop than the controls (6.5 $\pm$ 28.1) Figure 3.2. However, the relative effects of OA treatment on mite drop also varied among dates (Treatment*Date) (F=20.87; d.f. 6, 170; p=0.001). Separate analyses for each date showed significant treatment effects within dates Figure 3.2 with both OA treatments showing greater mite drop than the controls on most fall dates.

Overall analyses of the main effects of OA treatment, and wintering method in the period after wintering treatments were applied indicated that OA treatments also affected overall mite drop (F=3.74; d.f. 2, 53; p=0.0302) but there were no overall effects of wintering method on mite drop (F=3.33; d.f. 1, 53; p=0.0735). Relative treatment effects did vary with date in the case of both OA treatments (Treatment*Date) (F=5.88; d.f. 10, 224; $p < 0.0001$), and wintering methods (F=7.50; d.f. 10, 224; $p < 0.0001$). Therefore, differences in means among both of those treatments were examined with separate analyses by date. The three way interaction between OA treatment wintering method and date was not significant (F=0.45; d.f. 10, 224; p=0.9206). In the winter period mite drop on boards was highest in untreated colonies on all dates except 14 December (Figure 3.3) . The treatment that had received four applications of OA had less mite drop than controls on most dates from January onward (Figure 3.3) and the treatment that had received only one application of OA had lower mite drop than controls only after bees were removed from winter quarters (2 April). That treatment had intermediate levels of mite drop between four applications of OA and the untreated controls and did not differ from other treatments on all other dates (Figure 3.3). Differences in mite drop between wintering methods were significant only on 31 Jan (F=9.03; d.f. 1, 53; p=0.0041) and 5 March (F=14.51; d.f. 1, 53; p=0.0004) with significantly more

mite drop in indoor wintered colonies than in outdoor wintered colonies on both of those dates (Figure 3.4).

Cluster Size

On the 12 of November prior to treatment with OA population sizes were similar in the three OA treatment groups (F=1.08; d.f. 2, 54; p=0.3032). Although randomly assigned to future wintering treatments there was an interaction between OA and the wintering block groups (F=4.12; d.f. 2, 54; p=0.0216). Further analyses using single degree of freedom contrasts separating wintering blocks indicated there were no significant differences in the effect of OA treatments within either indoor groups (F=2.95; d.f. 2, 54; p=0.0609, slice) or outdoor wintered blocks (F=1.78; d.f. 2, 54; p=0.1783, slice). In contrast on the final inspection before movement to wintering sites (indoor or outdoor wintering) there were significant overall effects of OA treatment on population size (F=3.41; d.f. 2, 53; p=0.0404) with colonies treated with four applications of OA having greater cluster scores than those not treated with OA Figure 3.5. There was also an interaction between wintering block and OA treatment with (F=4.45; d.f. 2, 54; p=0.0163) with the OA treatments increasing population scores relative to untreated colonies more in the indoor block (F=7.28; d.f. 2, 54; p=0.0016, slice) than in the outdoor block (F=0.56; d.f. 2, 54; p=0.5755, slice).

In spring (April 2nd) neither the proportion of colonies surviving ($X^2 = 0.61$; p=0.737) nor population size of the surviving colonies were affected by OA treatment (F=1.15; d.f. 2, 34; p=0.3293). Wintering method did not affect the cluster size of surviving colonies (F=0.0; d.f. 1, 34; p=0. 9598) but overall survival was greater in colonies wintered outdoors than indoors ($X^2 = 4.54$; p=0.0331) (Supplemental Figure 3.17) and there was no interaction between wintering method and treatment for cluster size (F=1.17; d.f. 2, 34; p=0.3230) or colony survival ($X^2 = 2.51$; p=0.2857).

Colony Weight

OA treatments did not have a significant effect on the final colony weight prior to the addition of supplemental feed (F=1.87; d.f. 2, 56; p=0.1642) or after feeding (F=1.34; d.f. 2, 56; p=0.2696). Pre feeding weights on 27 November, 2020 ranged from 29.0 ± 0.8 to 31.1 ± 0.8 Kg and post feeding rates ranged from 30.4 ± 0.6 to 31.8 ± 0.6 Kg. In Spring (on 1 April, 2021), there was an effect of OA treatment on colony weight (F=3.19; d.f. 2, 56; p=0.049). Colonies treated with 4 applications of OA had lower weights (20.8 ± 0.7 kg per hive) than colonies treated with either 1 application of OA (22.7 ± 0.7 kg per hive) or those left untreated weights (23.3 ± 0.7 kg per hive) ($P < 0.05$, Tukey). Wintering method had no effect on final colony weights (F=0.72; d.f. 1, 53; p=0.3995).

Viruses

Neither OA treatment (F=0.82; d.f. 2, 35; p=0.4475) nor wintering method (F=2.09; d.f. 1, 35; p=0.1571) affected the abundance of virus in worker bees when looking at their pooled effect on all viruses. However, there was an interaction between wintering method and virus type (F=5.08; d.f. 4, 35; p=0.007) Figure 3.6. Virus abundance of DWV-A was greater in outdoor-wintered colonies than in indoor-wintered colonies (F=16.63; d.f. 1, 35; p=0.0002), concentrations of virus were marginally greater in indoor wintered colonies than in outdoor-wintered colonies for IAPV (F=3.86; d.f. 1, 35; p=0. 0.0574) but did not differ between wintering environments for other viruses tested, DWV-B (F=1.06; d.f. 1, 35; p=0.43105), BQCV (F=0.01; d.f. 1, 35; p=0. 0.9383) and SBV (F=0.03; d.f. 1, 35; p=0.8709) Figure 3.6.

Analyses by individual viruses showed only a marginal effect of OA treatment on DWV-A (F=3.18; d.f. 2, 35; p=0.053) with 1 application of OA having lower DWV-A than 4 applications (p=0.0425, Tukey-Kramer) but not differing from the control (p=0.2748, Tukey-Kramer) Figure 3.7. And

4 applications of OA did not reduce OA level relative to control (p=0.5703, Tukey-Kramer). Oxalic treatment did not affect the abundance of BQCV (F=0.76, d.f. 1, 35; p= 0.4773), DWV-B (F=0.76, d.f. 1, 35; p= 0.4773), IAPV (F=0.76, d.f. 1, 35; p= 0.4773) or SBV (F=0.76, d.f. 1, 35; p= 0.4773).

Experiment 2:

Mean Abundance of varroa mites

The combined effect of date and initial mite level on varroa abundance is shown in Supplemental Figure 3. 18**.** The mean abundance of varroa mites increased with time, even in treated colonies ($F = 38.43$, $df = 2, 61$; $p < 0.001$). As expected, there were more mites in the high initial mite block than in the low initial mite block ($F = 16.52$, $df = 1, 48$; $p = 0.0002$) and this trend did not vary significantly over time (mite-block*time $F = 0.85$, $df = 2, 61$; $p = 0.4325$). There was no overall effect of oxalic treatment ($F = 0.93$, $df = 2, 48$; $p = 0.4019$) or wintering environment ($F = 0.1$, $df = 1, 48$; $p = 0.9985$) on mean abundance of varroa and no interactions with each other or over time ($P > 0.05$).

Daily mite mortality rate

DMMRs of treated and control colonies were compared on each day the sticky board was collected. The 5-day interval treatments began on October 19, 2021. Prior to treatment, sticky boards were placed in the hives for 22 days, from September 27 (day 0) to October 19 (day 22). During this pre-treatment period, a significant difference in mite mortality was observed ($F =12.00.$, $df = 1, 26$; $p =0.0019$) , with the colonies that later underwent 5 day interval treatment having a slightly lower mite mortality rate (0.003 ± 0.0007 (proportion of mites dying per day) compared to the colonies that were assigned to become controls (0.007 ± 0.0007) shown in Supplemental .

The first 5-day oxalic treatment was applied on October 19, and the sticky boards were replaced, on October 24 (day 27). At this point, there was the effect of block ($F = 8.37$, $df = 1, 26$; $p = 0.0076$), mite mortality was low (0.006 ± 0.002) in colonies with initial low mites as compared to high mite group (0.01 ± 0.002). On November 3 (day 37), there was a significant effect of block ($F = 9.82$, $df = 1, 26$; $p = 0.0042$), mite mortality was low (0.019 ± 0.006) in colonies with initial low mites as compared to high mite group (0.048 ± 0.006).

The 4th (final) application of the 5-day interval treatment was on November 3, 2021, and for 12-day interval treatment was on November 2, 2021. Sticky boards from all colonies were collected on the same dates from this point forward, starting from November 9, 2021. Data were analyzed by day and block. In the high mite block, A significant effect of OA treatment on mite mortality rate was observed on (day 43) November 9 ($F = 5.99$, $df = 2, 25$, $p = 0.007$), where colonies treated with the 5-day interval OA treatment exhibited a higher DMMR (T5) (0.05811 ± 0.006575) compared to the control group (T0.12) (0.02651 ± 0.006575) shown in Supplemental Figure 3. 19.

In preparation for the transition to their respective wintering methods, colonies were moved from the apiary at the Glenlea Research Station, Mb to the U of M campus apiary on day 43 (November 9), and new sticky boards were placed in the colonies from November 9th to 12th. For the high initial mite abundance block, colonies treated at the 12-day interval exhibited a marginally higher mite drop (T12) (0.04538 ± 0.007864) than the control colonies (T0.12) (0.01603 ± 0.008289) on day 46 (November 12, 2021) ($F = 3.3$, $df = 2, 25$, $p = 0.053$) (Figure 3.8 A).

Sticky boards were also collected monthly during winter. In the low initial mite block, the untreated controls (0.0246 ± 0.0053) showed higher mite mortality than the colonies that had been treated at 5-day (0.0068 ± 0.0058) intervals on day 172 (March 18, 2022) ($F = 3.71$, $df = 2, 25$, $p = 0.038$).

Additionally, In low block, there was a significantly higher DMMR in the control colonies (T0.5) (0.01903 ± 0.003544) compared to the colonies treated at 5-day intervals (T5) (0.002112 ± 0.002893) when colonies were moved out of the wintering building on day 207 (April 22, 2022) ($F = 6.88$, $df = 2, 17$, $p = 0.0065$) (Figure 3.8 B).

Colony Survival and Spring Cluster Size

When survival data was analyzed, the interaction of miteblock*OA-treatment interaction was significant ($DF = 2$, $\chi^2 = 6.32$, $p = 0.0424$) with differences in the proportion of colonies surviving observed only within the Low block, OA treatment increased colony survival ($DF = 2$, $\chi^2 = 6.14$, $p = 0.0465$). Contrast analysis within this group the 5-day OA treatment increased survival relative to the control group ($DF = 1$, $\chi^2 = 5.84$, $p = 0.0157$) but the 12-day OA treatment did not ($DF = 1$, $\chi^2 = 0.22$, $p = 0.6401$) Figure 3.9. For colonies that survived the winter, there was no effect of treatment on cluster size. Although, the cluster size of colonies was reduced significantly from November to the following April ($F= 67.27$, $df = 1,19$; $p < 0.001$) (Supplemental Figure 3. 20).

Colony Weight

As expected, all living colonies lost weight over time ($F = 247.19$, $df = 1, 46$; $p < 0.001$), average colony weight before wintering was 32.6 kg (+/- 0.4) (2021/11/17) and reduced to 13.4 (+/- 1.2) kg in spring (2022/04/27). There was a significant 3-way interaction between wintering environment*initial mite level*date ($F=7.56$, $df=1,46$; $p=0.0085$). When looking at the effect of the wintering environment and mite level separately, interaction was present within the low mite level ($F=4.71$, $df=1,24$; $p=0.04$) and indoor wintering environment ($F=14.57$, $df=1,22$; $p=0.0009$). When the colonies were wintered indoors, the colonies with low initial mite levels lost more weight over winter (Figure 3.10).

Total Protein

There was an overall effect of date (F = 8.48, df = 2, 60; p = 0.0006) on the level of protein in worker bees (Figure 3.11). The total protein level in workers was similar between the September and November sampling dates but decreased significantly in colonies that survived until April. There was no overall effect of OA treatment (F = 1.23, df = 2, 48; p = 0.3011), initial mite level (mite block) (F = 0.69, df = 1, 48; p = 0.4093) or Wintering Environment (F = 0.16, df = 1, 48; p = 0.6937) on total protein. However, the interaction between OA treatment and initial mite block (F=4.18, df= 2,48; p=0.021) and its effects on total protein was significant. (Figure 3.12). When the interaction was examined further, Comparisons within OA treatment levels indicated significant differences in protein only within the 12 day interval OA treatment where colonies exposed to the high intial mite load had lower protein than those exposed to a low initial mite load (F=4.22, df= 1,48; p=0.0453 slice). Comparisons within OA treatment levels indicated no significant differences in protein for the 5 day interval OA treatment (F=1.56, df= 1,48; p=0.2181 slice) and control (F=3.35, df= 1,48; p=0.0736 slice). We saw no differences among treatments in the low mite block (F=1.28, df= 2,48; p=0.2862 slice). However, OA did affect relative protein within the high mite block (F=4.15, df= 2,48; p=0.021 slice).

Virus

Viral levels fluctuated over time, showing different patterns for each virus (F =47.25., df = 4, 470; p <0.0001) (Supplemental Figure 3. 21). DWV-A (F =47.59., df = 2, 59; p <0.0001) and DWV-B (F =8.82., df = 2, 59; p =0.0004) abundance increased from September (2021/09/22) to November (21/11/08), followed by a decrease in April (2022/04/27). In contrast, BQCV (F =65.51., df = 2, 59; p <0.0001) levels decreased from September to November, then increased in April. IAPV (F =39.26, df = 2, 59; p <0.0001) levels steadily increased from September through April. SBV (F =9.75, df = 2, 59;

p=0.0002 <0.0001) levels decreased from September to November, with no significant difference observed between April and either September or November.

The three way interaction between the treatment*date*wintering environment was significant for DWV-B (F =3.43., df = 4, 59; p =0.0137). Therefore, we did separate analysis by date, and saw a significant interaction between OA treatment and block on 2021-11-08 (F =8.46, df = 1, 47; p =0.0401). When the interaction was further examined, we did not see a significant effect of OA treatment within the low initial mite level (F =1.16, df = 2, 47; p =0.3222 slice) or high initial mite level blocks (F =2.48, df = 2, 47; p =0.0945 slice). The overall effect of mite level (F =8.76., df = 1, 48; p =0.0048) was also significant for DWV-B , and viral abundance was less in the colonies with low initial varroa mites in comparison to colonies having high initial varroa mite levels Figure 3.13. However, this was not consistent within all treatments. Initial mite level influenced DWV-B levels within the 12 day oxalic treatment (F =4.41, df = 1, 47; p =0.0411 slice), 5 day oxalic treatment (F =9.79, df = 1, 47; p =0.003) but not within the control (F =0.12, df = 1, 47; p =0.7301) Figure 3.14. Within both oxalic treated groups the colonies with lower initial mite levels had lower DWV-B than those with higher initial mite levels (Figure 3.14).

The effect of the wintering environment (F =7.44., df = 1, 48; p =0.0089) was significant for DWV-B with lower viral abundance in the colonies which were wintered indoors than in the outdoor wintered colonies (Figure 3.15). For BQCV, IAPV and SBV treatment, initial varroa level or wintering environment were not significant.

Colony survival

In the late fall experiment, colony survival was significantly influenced by Varroa destructor infestation and colony weight in November. A backward stepwise logistic regression identified Varroa

infestation level ($\chi^2 = 4.49$, df = 1, p = 0.0338) and colony weight ($\chi^2 = 5.29$, df = 1, p = 0.0219) as significant predictors of overwinter survival (3.16). Variables such as DWV-A, DWV-B, SBV, BQCV, IAPV, cluster size, protein content, and Nosema levels were excluded from the final model due to non significance (p > 0.36). Colonies with higher Varroa levels in November were less likely to survive the winter, with an odds ratio of 0.823, indicating a 17.7% decrease in the likelihood of survival for each unit increase in infestation level. Similarly, colony weight had a strong effect on survival, with lighter colonies showing reduced survival rates (odds ratio = 0.010), suggesting that lower pre winter food stores or colony biomass may have compromised overwintering success.

Discussion

This study was conducted to examine the effect of 1g late fall OA sublimation treatments on varroa mite control and the health of honey bee colonies under varied treatment regimens and two mite infestation levels in two wintering environments. We first compared one or four applications (at 2 to 5-day intervals) to untreated controls where treatments were applied in mid-November. The second experiment compared four applications concentrated in late fall (mid Oct. to early Nov.) at 5-day intervals to applications spread out through mid to late fall (late September to early Nov.) at 12-day intervals between treatments. In both cases the final OA treatments were applied at the beginning of November. Although four applications of OA were more effective than one, efficacy was not consistently high under the highly variable environmental conditions during fall. The results highlight the high variation in long term efficacy of repeated 1 g doses of OA vapour and reveal the complex interplay of treatment frequency, environmental factors, and varroa and virus interactions on treatment outcomes. Although the efficacy of all fall treatment regimens with OA had low or inconsistent efficacy against varroa, four late applications with 1g were better than one and significantly improved colony survival but only when initial mite infestations were low. In the first experiment, we studied the impact of the number

of applications of `OA on efficacy and colony performance. Our treatments using 1g OA vapour applied to single brood chamber colonies in fall showed very low levels of efficacy against varroa regardless of the number of treatments or timing of multiple applications. Although OA caused significant mite mortality in some applications as evidenced from sticky board sampling, reductions in the mean abundance of mites on adult bees relative to controls were not observed in late fall in either year and showed significant reductions in mean abundance with four applications in only one of two years. Applications of one g OA at 2-5 day intervals in November showed greater mite mortality than controls but mean abundance was significantly reduced only in the spring. These findings are consistent with reports by (Gregorc et al., 2016) showed that the initial 3 applications of 1 g OA sublimation showed less than 23% varroa mortality, but the 4th application showed 76% varroa mortality (when brood rearing is limited). In contrast, other studies show good efficacy of OA treatments, as measured by alcohol wash with doses of only one gram per colony. (Al Toufailia et al., (2015) show even lower doses of OA than used in this study are effective in reducing varroa infestation (0.56 g showed 80.7 %) and similar doses (1.125 g) show 96.7% reduction in varroa infestation. Rademacher & Harz (, 2006), reviewed efficacy data from various studies and report78% efficacy for one application of 1 g OA and higher efficacy for three applications (90%) of OA. Doses of 1 g can also be effective under similar timings in fall in Northern climates. Work by (Bahreini et al., n.d.), show very high efficacy with a of 98% reduction for varroa in November trials in the Province of Alberta, Canada after 4 applications of oxalic when applied according to manufacturer’s guidelines using the same device under conditions similar to this study.

The use of different intervals between successive oxalic treatments could influence efficacy and affect colony performanc as treatments earlier in fall could provide long term benefits, it could affect the degree of exposure to mites that continue to emerge from protected sites under sealed brood, it could affect the concentration of treatment exposure to periods with no brood and could increase the chance of

some treatments occurring under more favourable winter conditions. Prouty et al. (2023) used mite fall to compare 4 treatments at 3-day, 5-day, and 7-day intervals using a 4g OA per vapor treatment. They collected data from sticky boards 72 hours after treatment, revealing that the 3-day and 5-day OA treatments resulted in higher mite fall compared to the control, but only after the first application. In contrast, the 7-day interval showed greater mite fall after the first three applications when compared to the control suggesting longer intervals are more beneficial but this may have been related to the amount of brood exposure. Gregorc et al. (2017), noted that repeated applications of OA, especially during broodless periods, increase mite mortality by ensuring that mites in adult bees are exposed to the treatment. Our study's results suggest that short intervals between subsequent OA applications can effectively increase mite mortality in the short term, but its long-term effectiveness in controlling mite populations remains unclear. In this study, treatment groups with 5-day or 12-day intervals between four applications of 1g OA both showed an increase in varroa mite abundance on adult bees in late fall that was similar in both treated and untreated colonies. These findings are consistent with those of Berry et al. (2022), who showed that in the presence of brood, even seven applications with 1g OA vapor treatment at 5-day intervals did not reduce the mite abundance (mites/100 bees) but rather maintained it at the same level. Similarly, (Jack et al., (2020, 2021) found that applying 1 g OA vapour to treat colonies one time or three times at 8-day intervals is not effective in reducing varroa whether applied in the presence or absence of brood. In our study brood area was not quantified but there would have likely been moderate to small patches of sealed brood in September and October during the beginning of 12 day interval treatments and very little to no brood in the later part of the 12 day treatments and during the entire 5-day treatment group so brood alone was not likely the reason for poor efficacy. Higher doses than the lable recommended amount that we used might be required. Jack et al. (2021) and (Al Toufailia & Ratnieks, (2018) both showed that a dose of 2-4 g OA vapour is effective in reducing mite infestation. We examined efficacy using both alcohol wash and stick board assessment methods. Although Jack et al.

(2021) and Al Toufailia et al. (2015) suggest that the better measure to test OA effectiveness is to look at the mite abundance rather than the mite fall, our results did not entirely support that conclusion as treatments that showed no effect on abundance but showed greater mite mortality on sticky boards did improve colony survival in one case. Prouty et al. (2023) Our study's results suggest that small intervals between subsequent OA applications can effectively reduce mite mortality in the short term, but its long-term effectiveness in controlling mite populations remains unclear. Extending repeated treatments at this dose over the entire fall period appeared to be of little benefit despite the exposure to more favourable temperatures.

This study analyzed results after each sticky board change and observed significant differences from the control in both years using two approaches. For the first experiment, only the number of mites that fell on sticky boards was counted as we had uniform assignment of mite levels and did quantify the total number of mites remaining in colonies at the end of the experiment. For the second experiment, the DMMR was calculated as in (Bahreini & Currie, 2015b; Underwood & Currie, 2007). A DMMR measurement is valuable for analyzing mite fall statistics across colonies with varying numbers of mites. The DMMR is a better measure than mite fall alone if the colonies mite levels are not equalized before treatments, because the colonies initially having higher mites will have a higher number of mites falling on the sticky boards, not necessarily because of the treatment effect. Mite drop differences would also be influenced by the unequal intervals between treatments in our experiment.

In both experiments mite fall or mite mortality was greater than the control within periods during (or several weeks after) treatments occurred and were less than the control in late winter. Experiment 1 revealed significant differences in mite fall between treatment groups. Before wintering, OA-treated colonies (both one-time and four-time applications) showed higher mite drops on sticky boards compared to untreated colonies. The effects of OA vaporization increasing mite drop in the fall are in line with

other studies (Rademacher & Harz, 2006). In this study, the effects of OA on mite drop were most notable from November 16 to November 23, with four applications leading to a significantly higher mite drop compared to the single application. In contrast, (Jack et al., 2020) observed no significant difference between mite fall of a single application or multiple (3) applications of 1g OA vapor but more brood may have been present in their colonies in Florida than in ours. During the winter months of the 2020-2021 experiment, the opposite trend in mite drop was observed, with untreated colonies showing higher mite drop on most dates in mid to late winter in comparison to four time treated colonies. A similar trend was observed for the DMMR for the 2021-2022 experiment, where, during wintering, lower mortality in the 5-day treated colonies was observed compared to both the control and the 12-day treated colonies at the end of the winter period. These low rates of mite drop/mite mortality in late winter are likely because there would have been fewer mites in the colony after OA treatment. So the lower natural mite drop, is likely related to natural mortality in the number of mites remaining in the colonies. The more effective OA treatments would leave fewer mites in the colony to fall off the bees and onto the sticky boards during the colder months.

Effects of fall oxalic treatment on subsequent impacts with different wintering methods have not been extensively researched. In this study, the indoor wintered colonies had higher mite drop or higher mite mortality in comparison to outdoor wintered colonies in both experiments. Similar results were observed by (Bahreini & Currie, 2015a), where they treated colonies with 1g Oxalic using a varox vaporizer on 13 November and observed more effective varroa control in indoor wintering environments than outdoor ones. These results collectively suggest that a benefit to indoor wintering may be that it allows for more effective extension of the effects of late fall OA in early winter than in outdoor wintered colonies at least in very in cold climates where outdoor wintered colonies may be more tightly clustered

and possibly impeding the distribution of acid to bees within the colony. This aspect requires further study.

Initial mite level also had an influence on the effectiveness of late fall oxalic treatments but was not consistent in both years. For the first experiment, the pre-treatment mean abundance of varroa mites was 3.5% whereas for experiment 2, for the low mite block, mean abundance was 0.2% and for high mite block was 2.4% and the spring results showed for low block 4% and for high block 7.6% For Experiment 2, the DMMR was greater than the control only within high mite block after the last/fourth application on the 9th to 12 of December. This was one week after the final treatments had occurred during the 5-day treatment interval and thus could have been because of residual effects of the cumulative OA treatments. Mite mortality within the high initial mite block for the 12-day interval treatments also showed higher DMMR than controls, but this result may have been influenced by the movement of colonies while transporting them from their fall apiary site to their wintering location. It is possible the act of moving colonies helped to better circulate oxalic in the colony as a result of bees breaking their clusters and increasing movement within the colonies, resulting in more effective exposure of varroa to the acid. Others have shown efficacy of acaricides can vary with mite level. Ostermann & Currie, (2004), showed that formic acid was more effective in colonies having low mite levels.

Low temperatures during application are likely to have played a factor affecting OA efficacy in our experiment. (Rademacher & Harz, (2006), recommend the outside temperature be higher than 2° Celcius for vaporizing OA. In the first experiment in this study, temperature ranged from -11° to -3° Celsius during the time of oxalic application on days when treatments took place. In the second experiment, the first two applications were applied on days with temperatures of 15° and 11° celsius, however, and the last two applications were applied at 0° Celcius. For the 5-day application treatment, the temperatures for subsequent applications were 5°, 1°, 4° and 0° Celcius. At lower temperatures, the

activity of honey bees would be less and bees would form a tight cluster, which could reduce the distribution of oxalic within the colony possibly leading to lowered exposure of mites and thus lower efficacy of oxalic. Temperature is not the only factor affecting efficacy in our experiment however, as (Bahreini et al., n.d.) had very high efficacy with four applications of OA in northern Alberta when temperatures are the time of application were also low on some dates (-12 to +6).

In the first experiment , 4 applications of OA treatment at 2-5 day intervals increased cluster size of colonies in November in comparison to untreated colonies. This is likely related to the higher rates of mite mortality and lower mite populations causing less impact on development of the development of the wintering honey bee population. However, the OA treatment did not improve spring cluster size or colony survival. Interestingly, although indoor wintered colonies had fewer mites and lower levels of DWV than outdoor wintered hives in both years the effects on colonies were mixed. Outdoor wintered colonies had greater numbers of colonies surviving the winter in the first experiment and there was no effect on cluster size or colony survival in the second experiment. Although survival was not greater for indoor wintered colonies, those wintered indoors with low mite loads lost relatively more weight over winter which is expected in colonies with greater cluster sizes that consume more stored feed. It is possible some of the colonies with high cluster sizes wintered indoors died from starvation just before removal from the wintering building as this move from the building occurred much later than normal (27 April) and this would potentially bias the results. When under stress, indoor wintered colonies often have greater survival than outdoor wintered colonies (Bahreini & Currie, 2015a; Desai & Currie, 2016b). As expected, all cluster sizes were significantly reduced after winter which is typical in both indoor and outdoor wintering where bees die and clusters shrink due to stressors like cold temperatures, mites, and viruses, other pathogens (Desai & Currie, 2016a). It is important to note that despite having relatively low efficacy, OA treated colonies exposed in late fall at 5-day intervals showed significantly higher

survival rates than either 12-day and untreated controls but only when initial mite levels were low. Although multiple applications of OA could potentially could have negative effects. Tellarini Prieto et al., (2024), repeated applications of OA vaporization at up to 20 times the recommended label dose and evaluated their effects on colonies. They found that colonies treated with 20 g OA exhibit an increase in adult worker bee mortality compared to controls, with a non-significant 23% decrease in brood area, but with no notable impacts on queen performance or sperm viability. Although, these findings suggest that honey bee colonies can tolerate elevated OA doses without substantial long-term effects on queen or brood health, higher doses still pose short-term risks to adult bees. However, the doses and frequency used in our study appeared safe, with no evidence of short-term negative impacts on colony health.

Since vitellogenin makes up the majority of the overall protein content in honey bees, levels of this lipoprotein are directly related to the health of honey bees (G. V. Amdam et al., 2003). Their bodies store these proteins so they can feed their young and survive the winter. Protein levels were not measured in Experiment 1. In contrast, protein levels in Experiment 2 rose slightly between September and November and declined over winter. These outcomes are consistent with those of (Kunc et al., 2019), who found that total protein levels rose between June and October. This rise in total protein is likelythe result of bees entering their wintering phase in September when they need less protein to feed their brood. Since the mean abundance of varroa mites was higher in the spring, it is possible that the low protein levels during spring were caused by the mites feeding on workers. Alternatively, the workers may be utilizing these proteins over winter to survive and to provision a new generation of bees during winter brood rearing, which could explain the decrease in spring protein levels (Pirk et al., 2010; Zheng et al., 2014). There was evidence of the effect of mite levels on total protein. When starting mite levels were low, colonies treated with OA at 12-day intervals exhibited higher protein levels than colonies with high initial mite levels. This might be the result of a decrease in protein levels brought on by more mites feeding on

the hemolymph proteins and fat bodies of adult or developing workers . Colonies with low initial mite levels that were treated at 12-day intervals showed somewhat greater protein levels. Although not conclusive this may also be as a result of less mite induced stress, even if OA treatment itself had no direct effect on protein levels (Plamondon et al., 2024). On the other hand, in colonies treated at 5-day intervals, there was no change in protein levels in comparison to controls, which could be related to the timing of the 5-day treatment which began after most of the wintering bee population would be established (L. Harris, 2017). Between September and November, I saw no significant change in protein levels as a direct result of the oxalic treatment. The study by (Sagona et al., 2024), which found that vitellogenin levels rose only within the first 24 hours following oxalic therapy before returning to baseline levels 48 hours later, is in keeping with this conclusion.

For experiment 1, neither OA treatment nor the wintering method had a significant overall effect on virus abundance in worker bees. However, there was an interaction between the wintering method and virus type. For DWV-A, virus abundance in spring was higher in colonies wintered outdoors compared to indoors. Similarly, for experiment 2, the wintering environment affected DWV-B virus abundance, with colonies wintered outdoors having higher levels than those wintered indoors. This is in agreement with a study by (Desai & Currie, 2016a) who showed using a “generic” DWV primer that DWV levels were higher in outdoor wintered colonies. There were no significant differences for other viruses tested (DWV-B, BQCV, and SBV) however, IAPV, virus abundance in spring showed an opposite trend to that of DWV and was marginally higher in colonies wintered indoors than outdoors. Colonies exposed to multiple applications of OA showed higher DWV-A than colonies treated with a single application but did not reduce DWV-A levels as compared to the controls. While this may suggest that multiple exposures to OA indirectly increase DWV-A levels through sublethal as proposed by (Hatjina & Haristos, (2005) and (Sagona et al., (2024) it could be explained if colonies with the lower mite loads found in that treatment

could survive through winter despite having higher viral loads. Experiment 2 measured viruses levels before and after wintering and also showed no overall effect of OA treatment on virus levels, which is consistent with previous studies (K. C. Evans et al., 2022; Plamondon et al., 2024; Tentcheva et al., 2004). In this study, the primary factor influencing virus levels was time, as different viral patterns were observed over the course of the study. Specifically, DWV-A and DWV-B followed similar patterns; their levels were similar in Sept and April but did increase in November. A similar result was observed by (Plamondon et al., 2024) for DWV-B, which had the same levels of viral loads between Sept and April, but for DWV-A, they observed higher viral loads in April compared to Sept. BQCV levels decreased in the spring in our study, which contrasts with (Plamondon et al., (2024), who found stable BQCV levels between September and April. Tentcheva et al. (2004) observed viruses in the same year and found similar levels of DWV and BQCV in spring to fall. In my study, SBV levels were similar in Sept and April, which is consistent with results found by (Tentcheva et al., (2004). Overall, OA treatment did not significantly affect virus levels. Initial varroa mite levels did influence DWV-B, with colonies having low initial mite levels showing lower virus abundance. Several factors could contribute to the observed variations in virus levels. As there was a significant interaction between treatment, date, and wintering environment for DWV-B. After 4 applications, DWV-B virus levels in the 12-day treatment group were higher in high mite colonies and lower in low mite colonies. This could be because varroa is well known as a vector for transmitting DWV-B (Wilfert et al., 2016).

Neither wintering environment nor mite levels affected other viruses, including BQCV, IAPV, or SBV. Although varroa mites are strongly associated with the amplification and transmission of DWV (Martín-Hernández et al., 2012), their role in spreading other viruses such as BQCV, IAPV, and SBV is less well established and likely less significant (Y. Chen et al., 2004; Di Prisco et al., 2016). This may explain why changes in varroa mite levels influenced DWV dynamics more strongly than those of other

viruses in this study. This may be due to the association between DWV and varroa mites, as well as potential effects of OA on virus levels, but it is not clear how.

In the late fall (Experiment 2), colony survival was significantly influenced by both *Varroa destructor* infestation levels and colony weight as assessed in November. Colonies with higher infestation levels had a lower chance of surviving the winter, which supports the well documented negative effects of varroa on colony health and overwintering success. This aligns with findings from previous studies that have shown increased winter mortality in northern climates with higher mite loads (Borba et al., 2022; Currie et al., 2010; Dainat et al., 2012a; Guzmán-Novoa et al., 2010).

Late fall colony weight was also a strong predictor of survival. Although lighter colonies in November generally had lower predicted survival, supplemental feeding provided during winter may have improved survival for some low weight colonies, partially weakening the relationship between fall weight and actual overwintering outcomes. This suggests that colony nutritional status or resource availability leading into winter plays a critical role in overwintering success. Colonies capable of accumulating greater food stores likely had other attributes that provided the necessary energy reserves to sustain the bees throughout the long winter period.

Interestingly, virus levels measured in November did not significantly influence colony survival in this experiment, and were excluded from the final logistic model. This could indicate that in late fall, physiological and resource related factors such as colony weight and mite infestation may outweigh the immediate impact of viral loads on colony fate. Alternatively, the effects of viruses may manifest more prominently later in winter or spring, especially when combined with other stressors.

These findings highlight the importance of ensuring low *varroa* levels and maintaining adequate colony weight before winter. Management practices that support fall colony buildup and reduce mite

pressure, such as well timed OA treatments and supplemental feeding, may be essential for improving overwintering survival particularly in colder climates or in outdoor wintering environments. While multiple OA applications in late fall contributed to reduced mite levels in spring, they did not consistently improve cluster size or survival, suggesting that treatment effectiveness may depend on initial mite levels. The added benefit of indoor wintering in reducing DWV further emphasizes the value of an integrated approach to colony management.

Figures

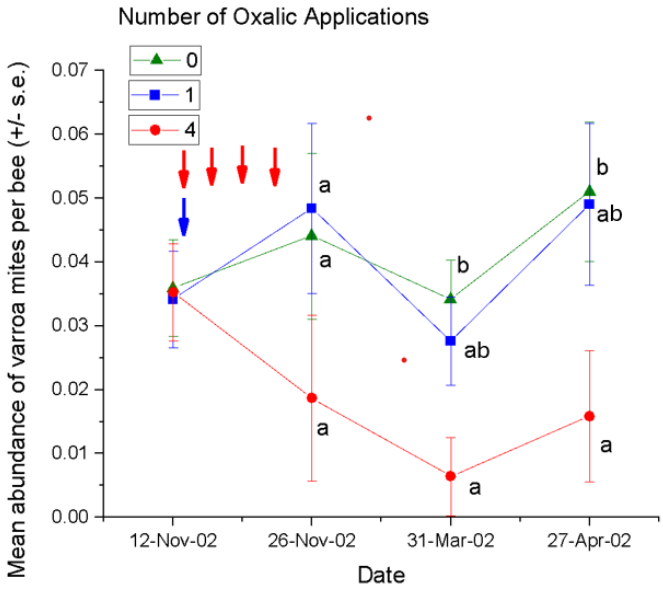

Figure 3.1: Effect of number of late-fall oxalic acid vapour applications on mean abundance of varroa mites (proportion of mites per bee) prior to treatment (on 12 Nov), following treatment prior to wintering on 30 Nov and following winter 31 March and 27 April 2021. Red arrows indicate timing of 4 applications of OA, Blue arrow indicates timing of 1 OA treatment. Colonies were treated with 1 application of OA (1), 4 applications of OA (4) or left untreated (0). Results for colonies wintered indoors or outdoors are pooled for analyses. Means followed by the same letter within dates are not significantly different (Tukey p ,0.05).

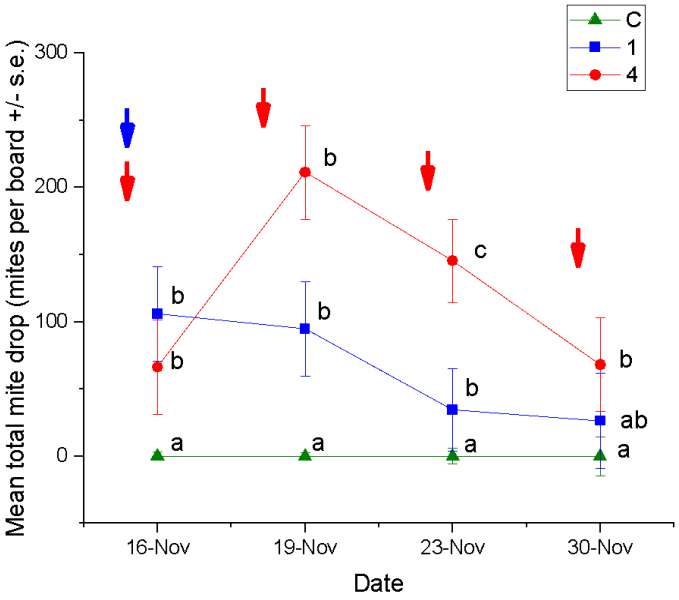

Figure 3.2: Effect of number of late-fall oxalic acid vapour applications on total mite drop per sticky board (mean number of mites per board +/- standard error). Dates reported are at the end of each sampling period prior to movement to wintering locations. Red arrows indicate timing of 4 applications of OA, Blue arrow indicates timing of 1 OA treatment. Colonies were treated with 1 application of OA (1), 4 applications of OA (4) or left untreated (C). Means followed by the same letter within dates are not significantly different (Tukey p ,0.05).

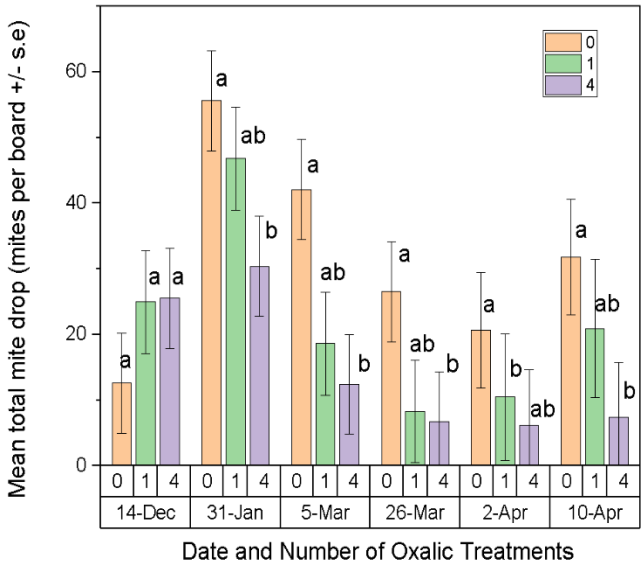

Figure 3.3: Effect of number of late-fall oxalic acid vapour applications on total mite drop per sticky board (mean number of mites per board +/- standard error) after colonies were moved into indoor or outdoor wintering locations. Dates reported are at the end of each sampling period following movement to wintering locations. Colonies were treated with 1 application of OA (1), 4 applications of OA (4) or left untreated (O). Means followed by the same letter within dates are not significantly different (Tukey $p < 0.05$). Indoor and outdoor wintering treatments are pooled for this analysis.

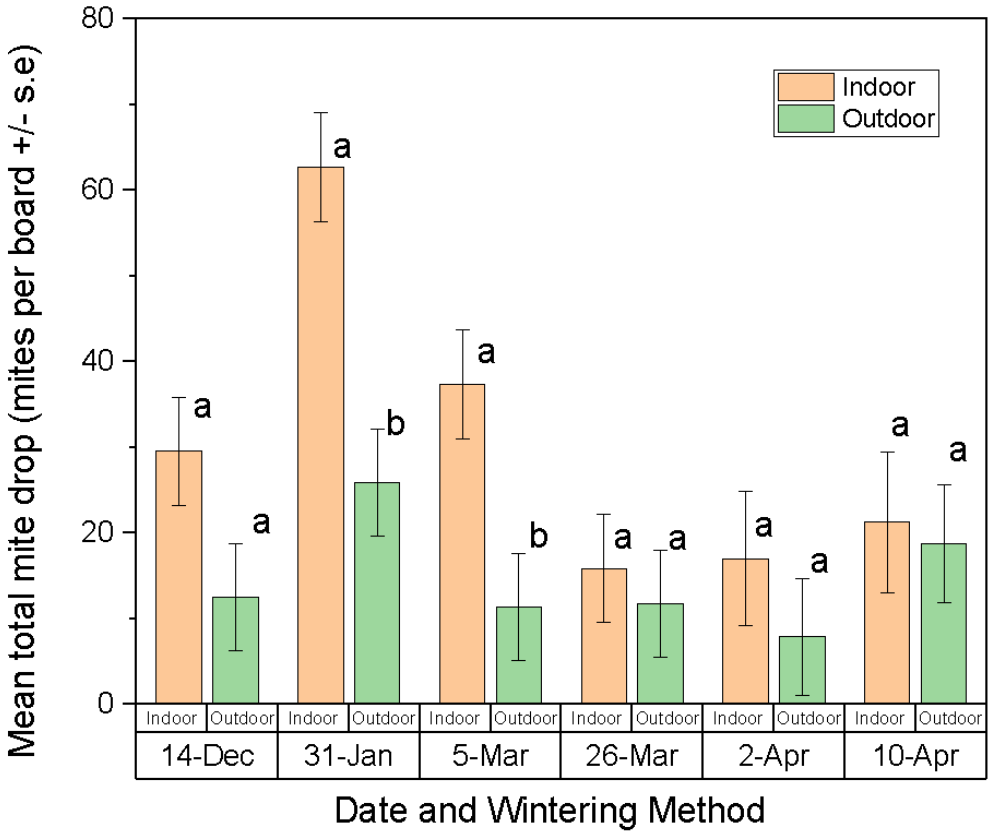

Figure 3.4: Effect wintering method on total mite drop per sticky board (mean number of mites per board +/- standard error) after colonies were moved into indoor or outdoor wintering locations. Dates reported are at the end of each sampling period following movement to wintering locations. Colonies were treated prior to being located in an indoor wintering facility or wintered outdoors using standard practice for the region. Oa treatments means are pooled for analyses. Means followed by the same letter within dates are not significantly different (Tukey p ,0.05).

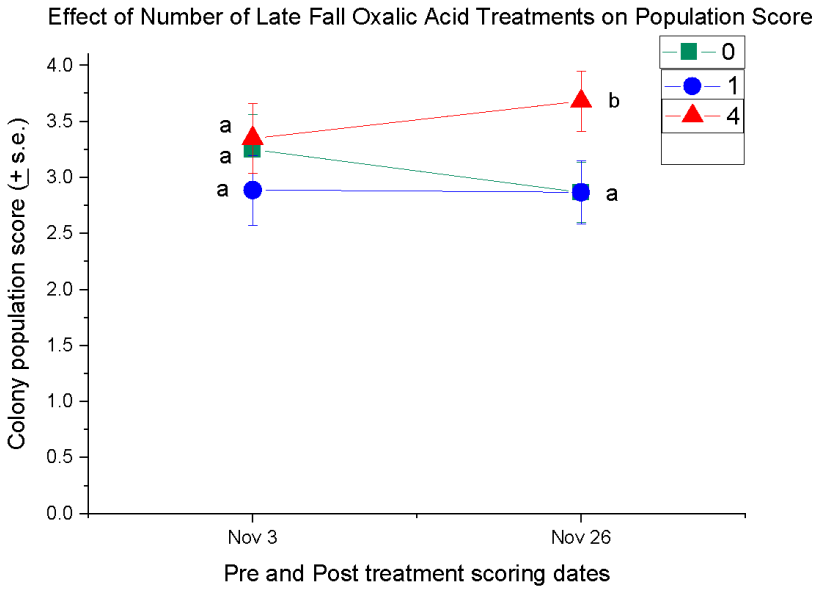

Figure 3.5: Effect of number of late-fall oxalic acid vapour applications on fall colony cluster scores (mean cluster score= frames of bees +/- standard error) prior to when colonies were moved into indoor or outdoor wintering locations. Dates reported are at prior to (3 Nov) or after the final OA treatment (26 Nov). Colonies were treated with 1 application of OA (1), 4 applications of OA (4) or left untreated (O). Means followed by the same letter within dates are not significantly different (Tukey p, 0.05).

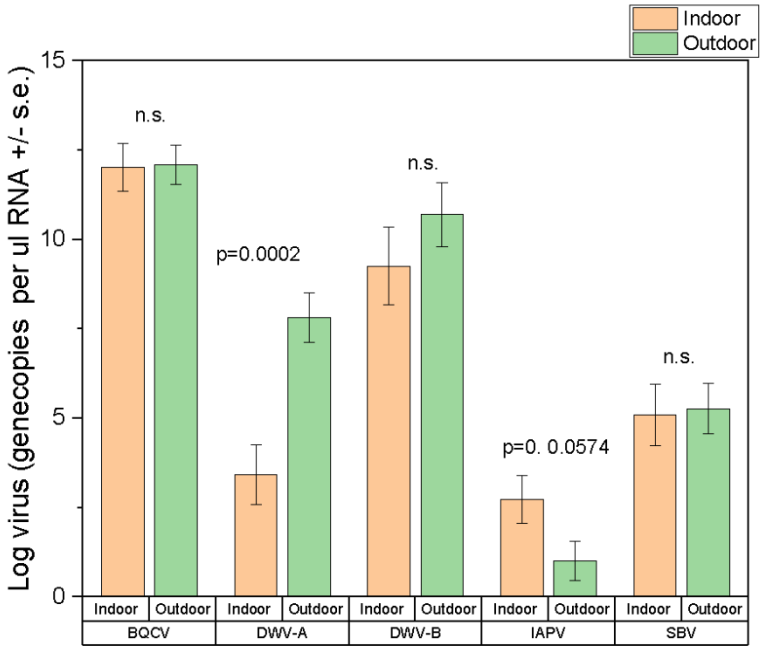

*Figure 3.6: Effect of wintering environment on virus mean abundance +/- standard error (gene copies /ul RNA) of black queen cell virus (BQCV), deformed wing virus variants (DWV-A and DWV-B), Israeli acute bee paralysis virus (IAPV) and sac brood virus (SBV) in adult workers sampled from colonies in spring. Data for all acaricide treatments are pooled for analyses. There was a significant wintering treatment*virus type interaction. Means followed by the same letter within dates are not significantly different (Tukey p ,0.05).*

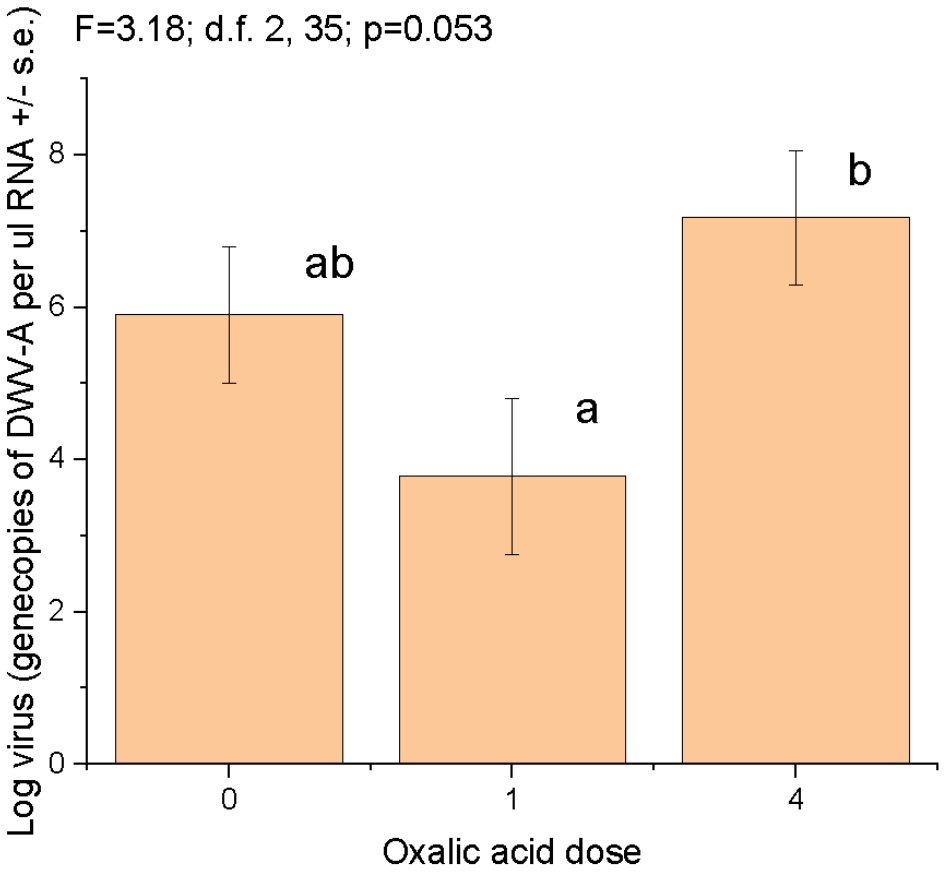

Figure 3.7: Effect of OA treatment on virus mean abundance +/- standard error (gene copies /ul RNA) of deformed wing virus (DWV-A) sampled from colonies in spring. Data for both wintering method treatments are pooled for analyses. Means followed by the same letter within dates are not significantly different (Tukey p ,0.05).

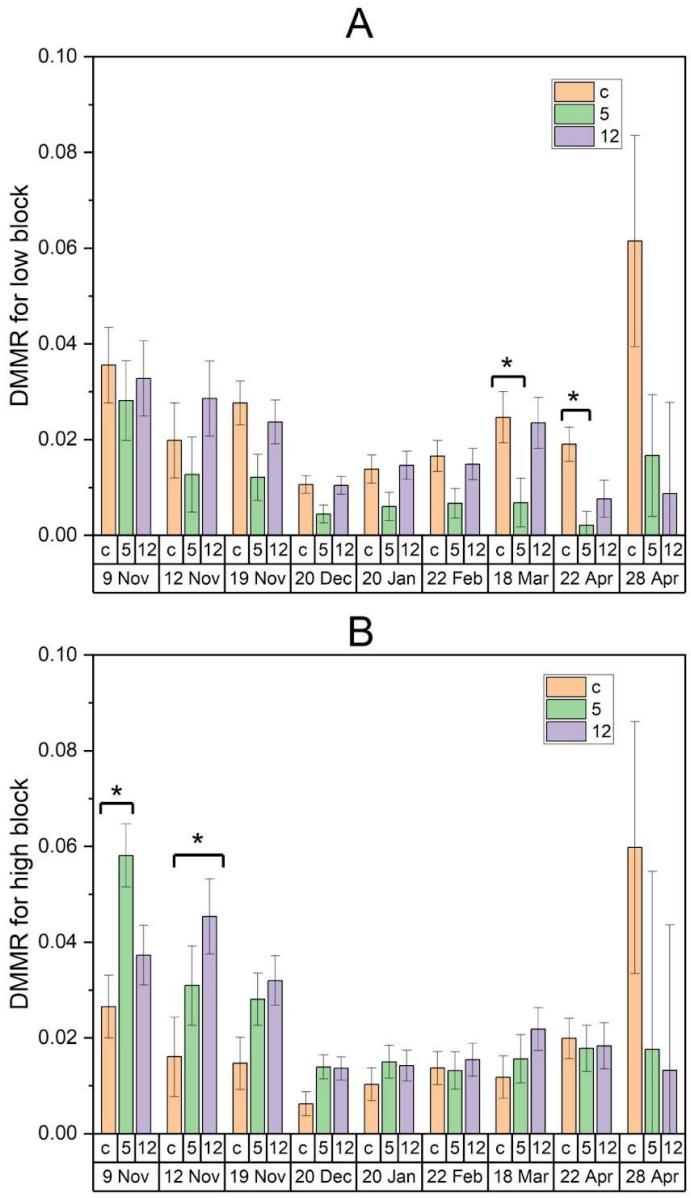

Figure 3.8: Effect of treatment on daily mite mortality rate (DMMR) (proportion of mites dying per day) from 9th Nov 2021 to 28th April 2022. The y-axis shows the DMMR, the bars represent means and the whiskers represents standard error. The x-axis shows the dates on which sticky boards were collected and the oxalic vapor treatment groups (untreated control (C), oxalic at 5 day interval treatment (5) and oxalic at 12 day interval (12)). Graph A is data for the low initial mite abundance block and graph B the high initial mite abundance block. Significant differences among oxalic treatment intervals within dates are marked with an asterisk (*) (Tukey p ,0.05).

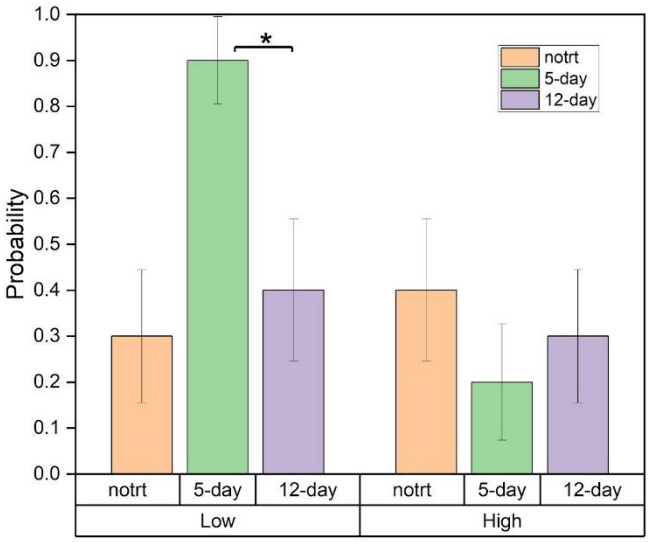

Figure 3.9: Effect of initial varroa levels and treatment intervals on survival of colonies in spring. The y-axis represents the probability of colonies surviving +/- standard error. Log linear analysis was used, Asterisk indicates significant difference between bars.

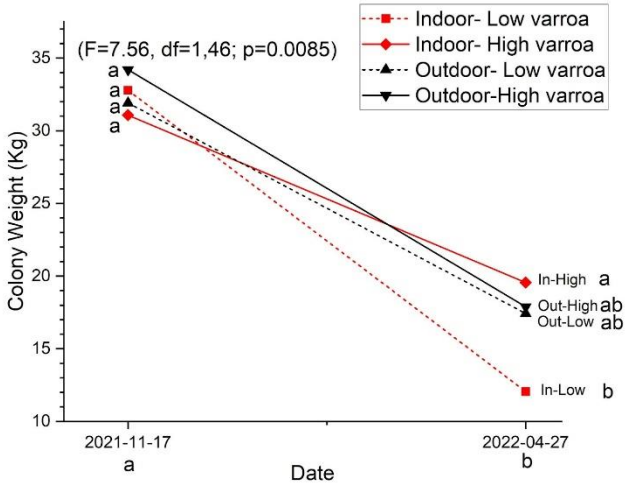

Figure 3.10: Effect of the interaction between wintering environment (indoor or outdoor wintering) and initial mite abundance (mite-block) and date on total colony weight (kg). The y-axis bars represent the mean colony weight in kg +/- standard error. The x-axis represents the date before and after the wintering environment. The red colour is for indoor wintering and block colour is for outdoor wintering. The initial mite abundance- Low (dashed line) or High (solid line) as determined by the mean abundance of varroa mites on 2021/09/22. The lines followed by the same letter are not significantly different from each other within each date.

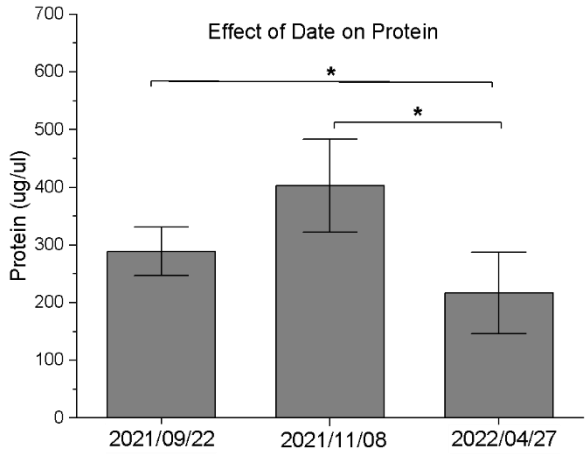

Figure 3.11: Mean total protein level (µg/µl) +/- standard error of homogenized bees by date. The lines connecting bars marked with an asterix (*) are significantly different from each other (Bonferroni P > 0.05).

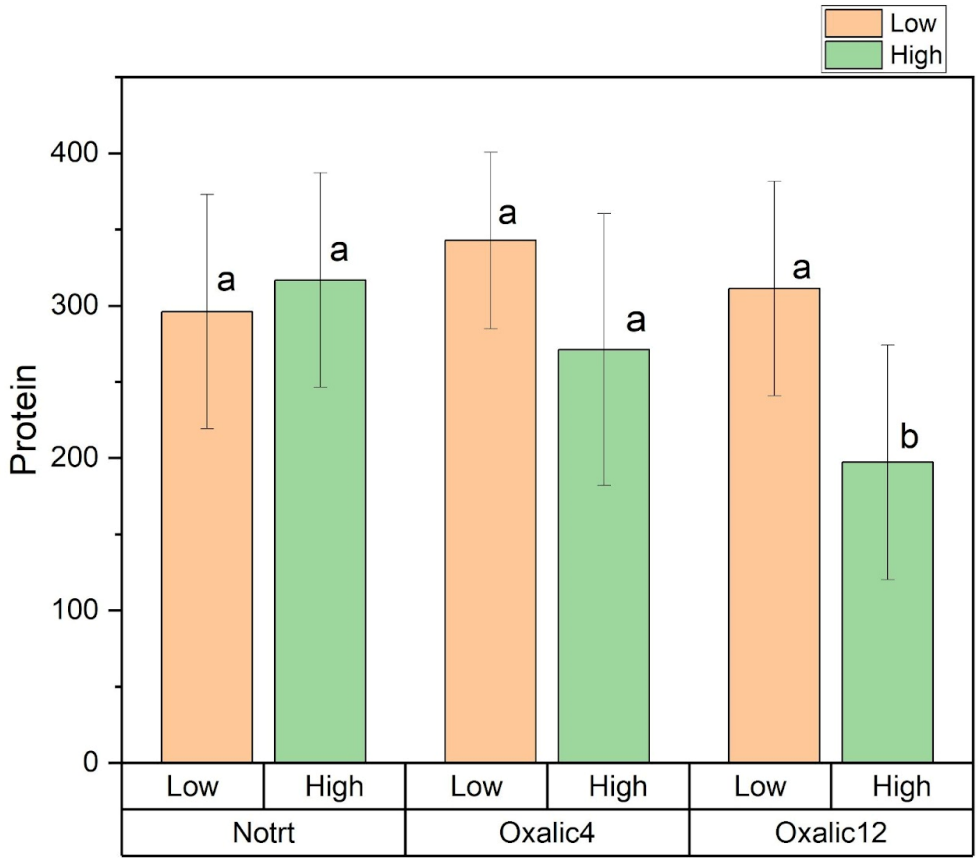

Figure 3.12: Effect of treatment and initial mite abundance (Low or High) on variation in total protein level of worker bees. The y-axis shows protein concentration +/- standard error and x-axis shows oxalic acid treatment group (untreated control (Notrt), oxalic at 5 day treatment interval (Oxalic5) and oxalic at 12 day interval (Oxalic 12)). Bars followed by the same letter within oxalic acid treatments are not significantly different from each other ($p < 0.05$).

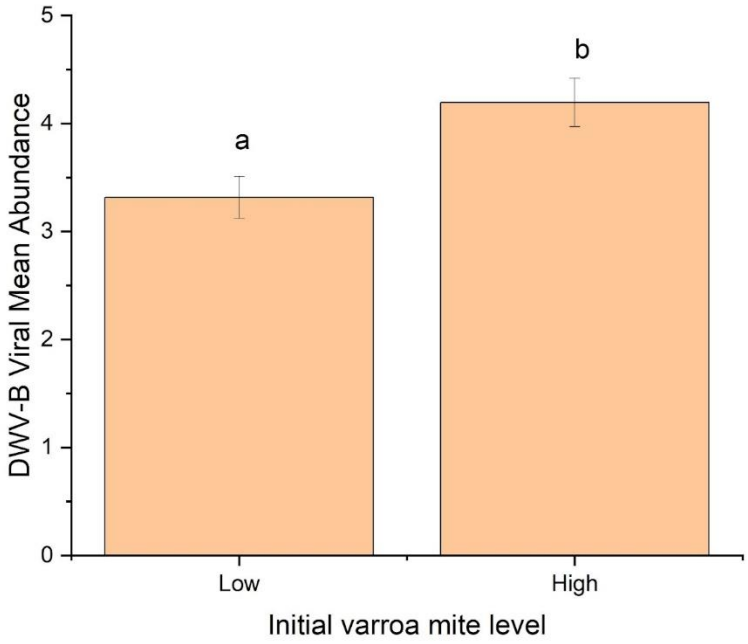

Figure 3.13: Effect of initial varroa mite abundance on mean abundance of DWV-B in spring. The y-axis represents log-transformed gene copies of DWV-B +/- standard error. The x-axis represents low and high varroa mite abundance. The means followed by the same letter are not significantly different from each other.

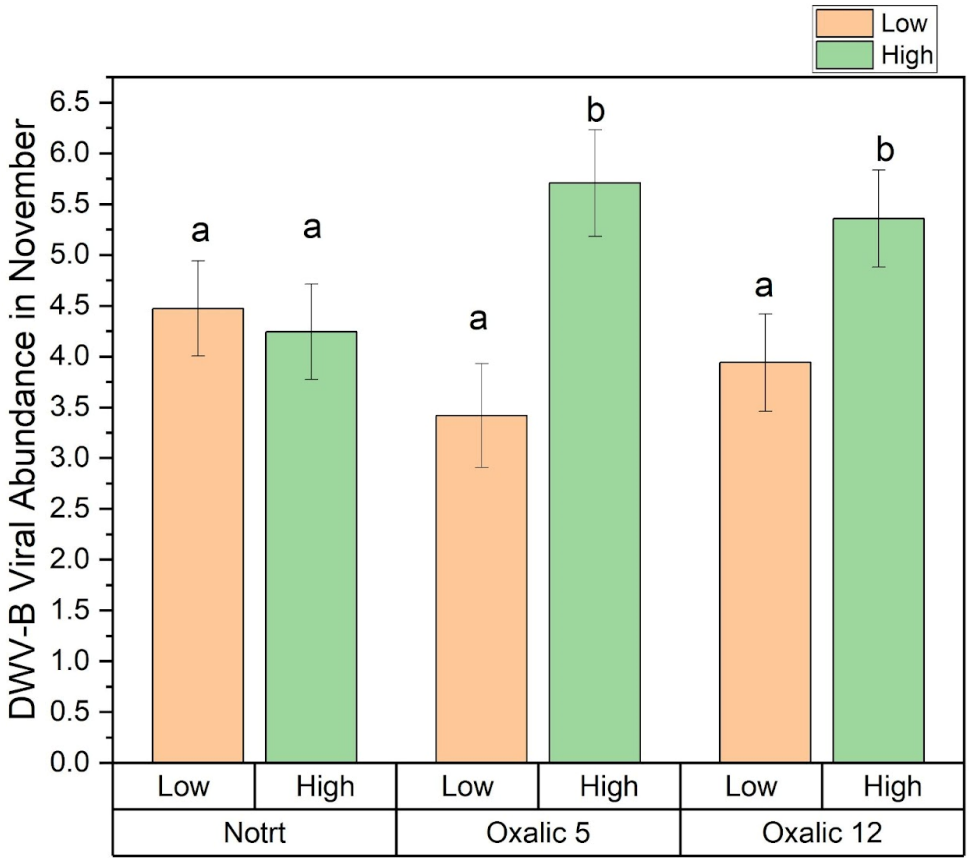

Figure 3.14: Effect of Interaction between oxalic treatment and initial mite block on mean abundance of DWV-B in November. The y-axis represents log-transformed genecopies of DWV-B +/- standard error. The x-axis represents the initial mite block (Low or High), which was determined by mean abundance of varroa mite on 2021/09/22 and Oxalic acid treatments (12 day interval, 5 day interval and no treatment). The means followed by the same letter within oxalic acid treatment are not significantly different from each other.

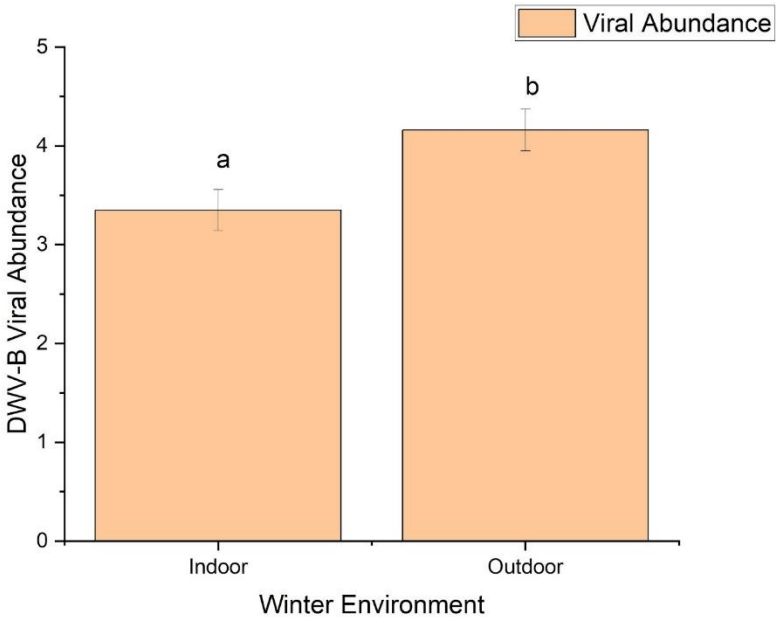

Figure 3.15: Effect of wintering environment on mean abundance of DWV-B. The y-axis represents log-transformed gene copies of DWV-B +/- standard error . The x-axis represents indoor and outdoor wintering environments. The means followed by the same letter are not significantly different from each other.

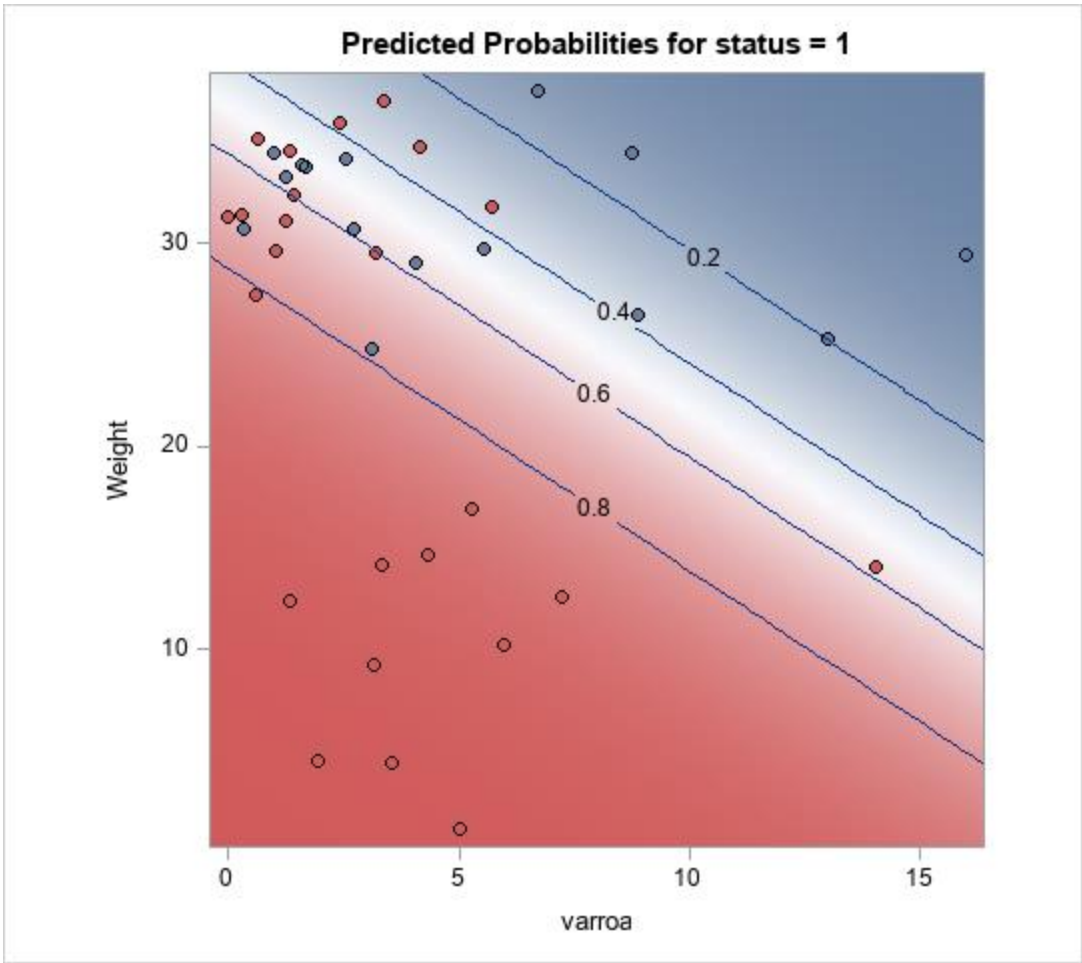

Figure 3.16: Fall predictors of colony mortality in spring. The analysis evaluated the effects of virus levels, Varroa infestation, and colony-level factors on overwintering survival using November data. Log-transformed colony weight and arcsine square root-transformed Varroa infestation levels were retained as significant predictors in the final logistic regression model. The graph displays untransformed values for interpretability.

Note: Colony weights reflect pre-winter conditions in November, before supplemental feeding provided during the winter months. This additional feeding may have helped some lighter colonies survive despite their lower fall weights, potentially influencing the predictive relationship between weight and survival.

Appendix

Target	G block
DWV-A	GTAGTTAAACCAATAAATGGTTGCAAGATTAGAAGTTTGCAAGATGCTATATGTGGTGTGCCTGG TTTAGATGGGTTTGATTCGATATCTTGGAATACTAGTGCTGGTTTTCCTTTGTCTTCATTAAAGCCACCTGGA ACATCAGGCAAGCGATGGTTGTTTGACATTGAGCTACAAGACTCGGGATGTTATCTCCTGCGTGGAATGCGT CCCGAACTTGAGATTCAATTATCAACGACACAGTTAATGAGGAAAAAGGGAATAAAACCTCACACTATATT CACGGATTGTTTGAAAGATACTTGTTTGCCTGTTGAAAAATGTAGAATACCTGGTAAGACTAGAATATTTAG TATAAGTCCGGTACAGTTTACCATACCGTTTCGACAGTATTACTTAGACTTTATGGCATCCTATCGAGCTGCA CGACTTAATGCTGAGCATGGTATTGGTATTGATGTTAACAGCTTAGAGTGGACAAATTTGGCAA
DWV-B	TCCGTTGAAGTTAGGGTGTGAGAAACATGGTATGCCATGTTCTCCATTTAATCGAAAACATTTGGA ATTAGCAACGACTCATTTAAAGGAGAAGTTAATTTCCGTAGTTAAACCTATAAACGGATGCAAGATTAGAA GTTTGCAAGATGCTGTGTGTGGTGTACCAGGTTTGGATGGCTTTGATTCAATATCCTGGAATACTAGTGCTGG TTTTCCTTTATCTTCATTAAAACCGCCAGGCTCTTCTGGTAAGCGATGGTTGTTTGATATTGAATTACAAGAT TCAGGATGTTATCTTTTGAGAGGGATGAGACCTGAACTTGAGATACAGTTGACAACAACTCAGTTAATGAGG AAGAAGGGAATGAAGCCTCACACTATATTCACGGATTGTTTGAAAGATACATGTTTGCCTGTGGAAAAATGC AGAATACCTGGTAAGACTAGAATATTTAGTATAAGTCCCGTCCAATTTACGATTCCATTCCGA
IAPV	AGGGTGAGGAGCCTCGGTGGCAGCCCCACCAAATCCTCTATTGGATAGGAACAGCTGTACTGGGC AGTTACAGCAGTCGTATGGTAACACATGCGGCGTTTCGAAATACCATGCCTGGCGATTCACAACAAGAAAG CAATACTCCCAAGGTACACAACACGGAACTCGCTTCGTCCACTAGTGAGAACTCGGTTGAGACCCAAGAAA TCACAACCTTTCATGATGTGGAAACTCCAAATAGGATCGATACCCCCATGGCTCAGGATACTTCATCGGCTA GGAACATGGATGATACGCACAGTATTATTCAGTTTTTACAGCGCCCCGTTCTCATTGACAACATTGAGATCA TTGCTGGAACAACTGCCGATGCAAACAAACCCCTTAGTCGATATGTGTTAGATCAACAAAATTCCCAAA
BQCV-310	GAATTCGGAAACATTTTACTATAGTTCAGGTCGGAATAATCTCGATATAGCCACTTCACCTCCCAC CGTCAATCGCTATTATGCGGTTGGTGCGGGAGATGATATGGACTTTTCCATCTTTATCGGTACGCCGCCCTGT ATTCATGCATCTCAGACGGCCCAGTTTACCAAGATAAAACAAGGTAAAGTGTATGATTTGAGGTATGATCAG TACGACCCTTTTAGGGAAGTCCAGGACGGTACGGCGTTCCTCAATGCTCGTAGTATTGAGGATAGCGATTTG TTGTGAGCTCCTTTAGAGGGAGGGCTCACTTTATCTATTGCTTAATTCGGTAAGCCACAAATTTTTCTAAGTG TCATGAGTTTCTTCTCGGTTCTTCTCATGATTACTAATCGAACCGTGTGTAGAGTCAGAATGTTGTGGTTTAC GTTTCTTCTTGTTGCTTCGAGTTAAAGTAGTTACTGAGAAGGGTGTAGATTTCGTCAGAGC
SBV-335	GGTGGAAGCTGGGGATGATTTTGAGGTGTCTAACTTTTATGGGCCACCGACAGTAAAGACGAATG ATTGAACTACGCATTCTCTGATGAGCACGCTCGAGTTCAGATGGATGACAGTGTTGAAAGAGTATATGATGA GGGAAACCAGGTGTATTACTATCCTCCCCCTAAACCAGAGGGTTTTAGTTTGAACAATGTACGGACTTCGGT TAGTACGTTGTGTAATATGCTTGGAAAAGTAGTAACTCCTGAGCGCGCAATGAAAACAGCGTTGTGCGCAAC TCCTTATTTTGGATCAGCTTATATGACAGCTACTACTCTTGATGCTATAGGTGGTATGCAGAATACCGTTACG GGAGCAGCACAACAGCTCACGGCGTCCGTTGATGCGAGGCTAGAGCAACTGTCAGCTAAGTTTGGAGATTC AATTGACGTAATCACTACAGCGGTTAAGGAGGCTATAGGTAAAATTTCTTCTGGTATGTTTAAT

Appendix 3. 1:Sequences of g-block used to quantify viruses using real-time qPCR. Forward and reverse primers are highlighted.

Target	Forward Primer	Reverse Primer
DWV-A	TTCATTAAAGCCACCTGGAACATC	TTTCCTCATTAACTGTGTCGTTGA
DWV-B	TATCTTCATTAAAACCGCCAGGCT	CTTCCTCATTAACTGAGTTGTTGTC
BQCV-310	CCTGTATTCATGCATCTCAGA	GCAACAAGAAGAAACGTAAACCAC
SBV-335	TTGGAACTACGCATTCTCTG	GCTCTAACCTCGCATCAAC
IAPV	CCATGCCTGGCGATTCAC	CTGAATAATACTGTGCGTATC
Actin	AGGAATGGAAGCTTGCGGTA	AATTTTCATGGTGGATGGTGC

Appendix 3. 2:Forward and Reverse primers used in the thesis for quantification of viruses.

Supplemental Material

Experiment	Date	Event	Temperature (°C)
One	12 Nov 2020	Cluster Score	-8
One	13 Nov 2020	OA	-3
One	16 Nov 2020	OA	-8
One	19 Nov 2020	OA	-3

One	23 Nov 2020	OA	-11
One	26 Nov 2020	Cluster Score	-2
One	1 Apr 2021	Cluster Score	+4
Two	27 Sep 2021	12-day OA	+15
Two	9 Oct 2021	12-day OA	+11
Two	19 Oct 2021	5-day OA	+5
Two	21 Oct 2021	12-day OA	0
Two	24 Oct 2021	5-day OA	+1
Two	29 Oct 2021	5-day OA	+4
Two	2 Nov 2021	12-day OA	0
Two	3 Nov 2021	5-day OA	0
Two	17 Nov 2021	Cluster Score	-8
Two	27 Apr 2022	Cluster Score	+3

Table 3. 1: Environmental conditions at time of OA vapour applications

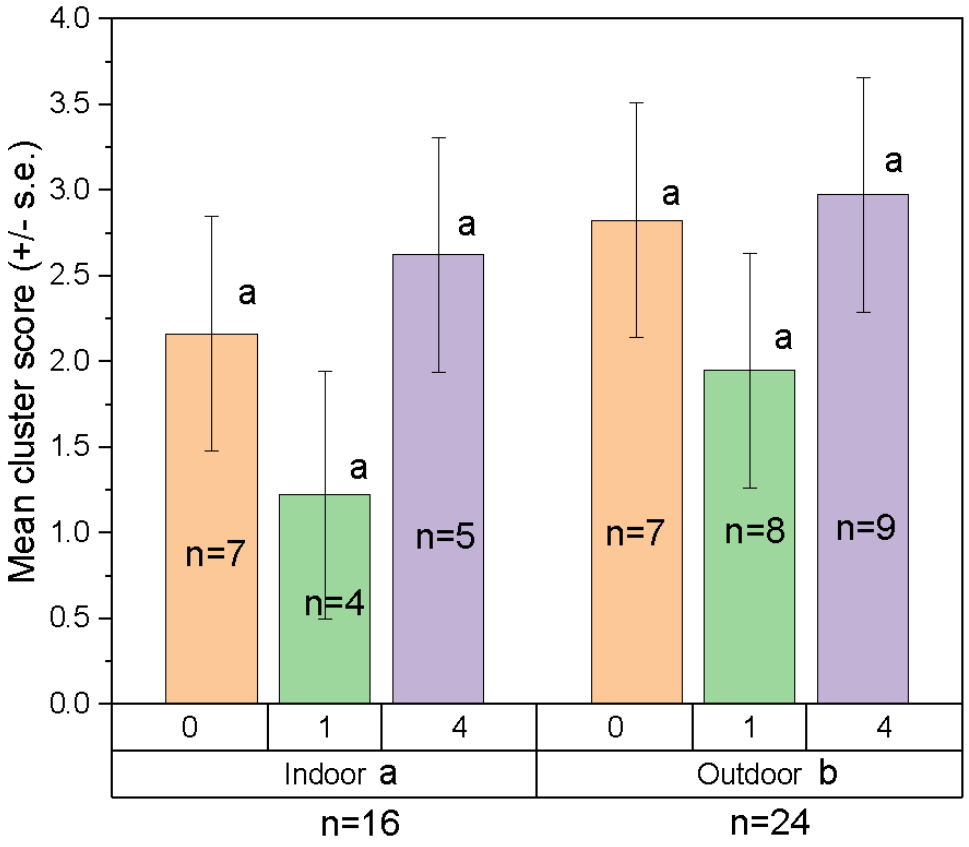

Figure 3.17: Effect of number of late-fall oxalic acid vapour applications on spring colony cluster scores (mean cluster score =frames of bees +/- standard error) and numbers of colonies surviving after colonies were moved from indoor or outdoor wintering locations. Dates reported are at the end of each sampling period following movement to wintering locations. Colonies were treated with 1 application of OA (1), 4 applications of OA (4) or left untreated (O). Means (Tukey $p < 0.05$) or proportions (logistic analyses) followed by the same letter within dates are not significantly different. N=number of colonies surviving out of 10 colonies for OA treatments within wintering methods and 30 colonies for wintering methods.

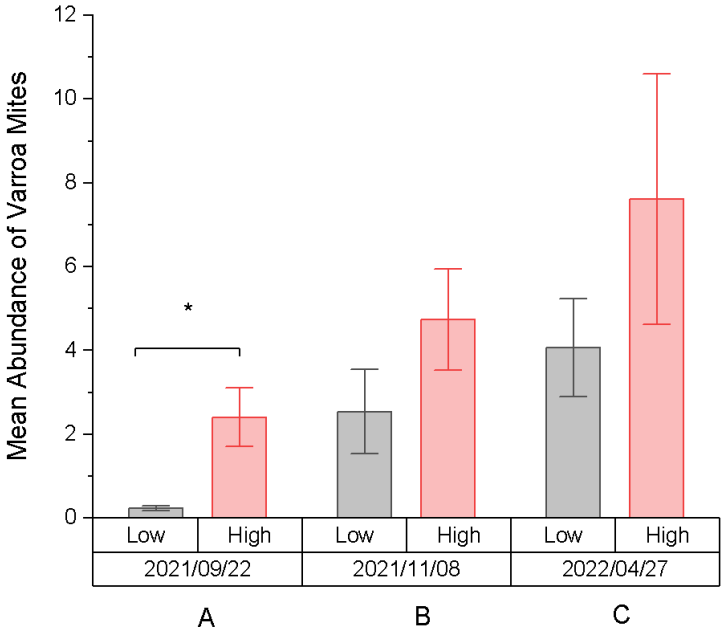

Figure 3. 18: Effect of the date and initial mite abundance (mite-block) on mean abundance of varroa mite. The y-axis represents the mean abundance of varroa (mites per 100 bees), bars represent means and whiskers represent standard error. The x-axis represents the date on which samples were collected, and the mite block (Low or High) as determined by the mean abundance of varroa mites prior to treatment on 2021/09/22. The mite-blocks marked with asterix (*) are significantly different from each other within each date (Tukey P > 0.05).

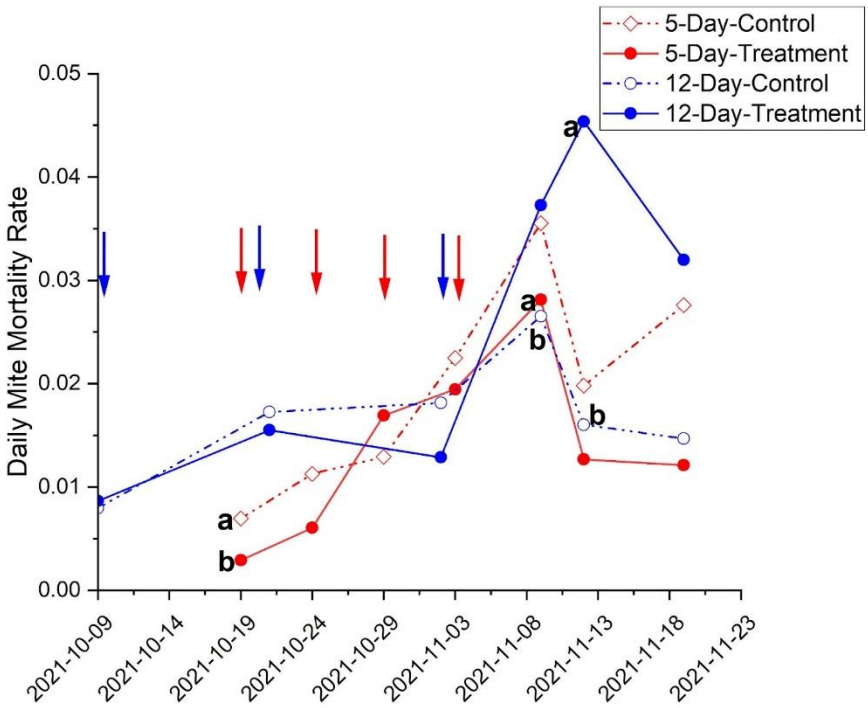

Figure 3. 19: Effect of Treatment on daily mite mortality rate (DMMR) (proportion of mites dying per day) from 27th Sep 2021 to 19th Nov 2021. The y-axis shows the DMMR +/- standard error, x-axis shows the dates on which sticky boards were collected. The arrows shows the dates on which treatments were applied. Points followed by the same letters within dates are not significantly different from each other (Tukey p ,0.05).

Note-Among the colonies analyzed for this graph, 12 day treatment and control colonies are from high initial mite group and all 5-day treatment and control colonies are from low initial mite group.

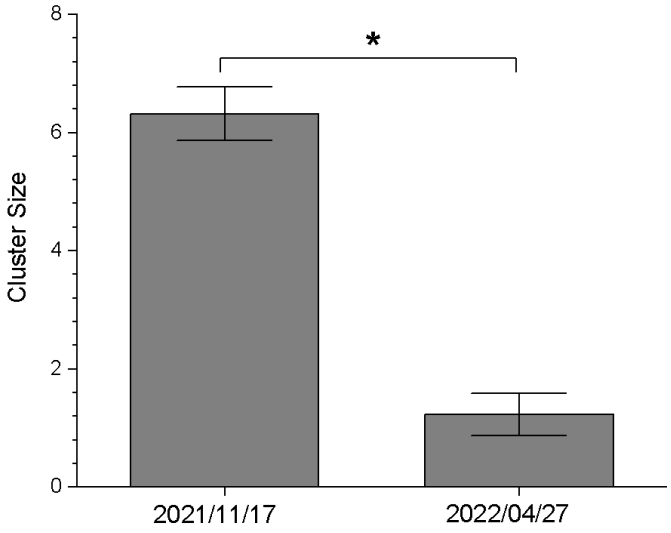

Figure 3. 20: The effect of sampling date on the colony's cluster size. The y-axis represents the cluster size of live colonies; bars represent the means and whiskers represent standard error. The x-axis represents the dates on which the cluster size was recorded. Bars marked with an asterisk (*) are significantly different from each other (Tukey P > 0.05).

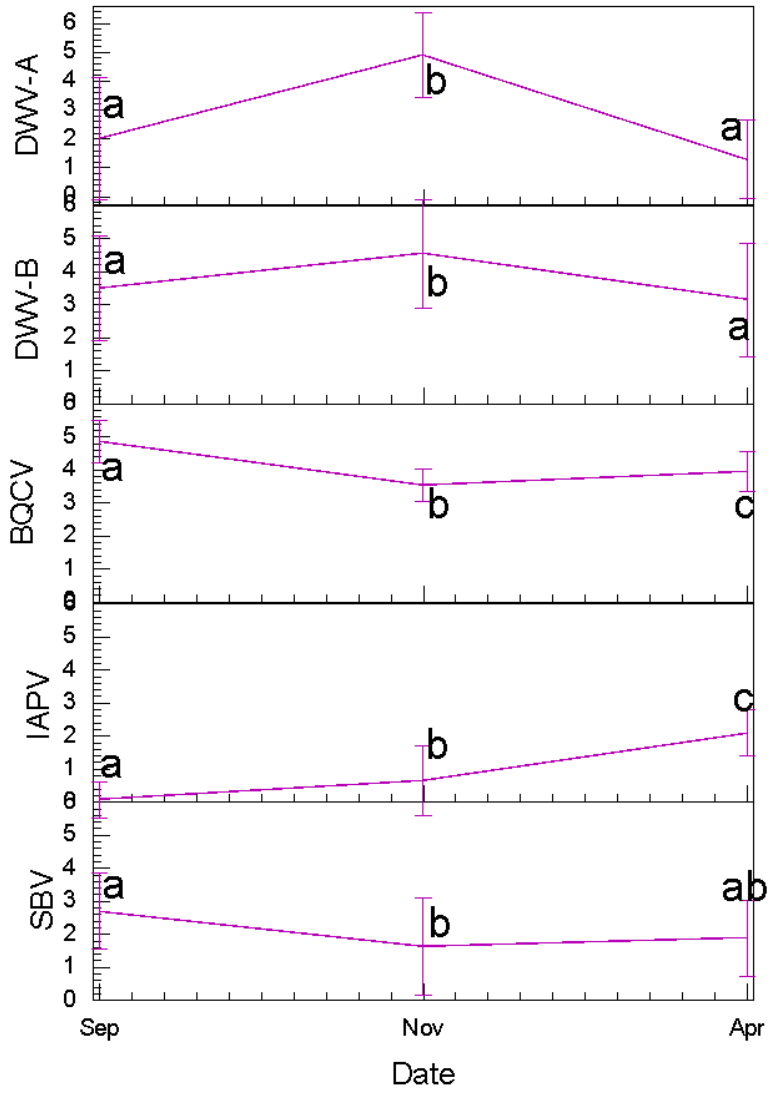

Figure 3. 21: The effect of date on the mean abundance of viral gene copies of DWV-A, DWV-B, BQCV, IAPV, and SBV in worker bees. The y-axis represents log-transformed viral genocopies and (+/-) standard error. The x-axis represents the dates (2021/08/22, 2021/11/08, and 2022/04/27) on which samples were collected. Within each virus points followed by the same letter are not significantly different from each other (Tukey P > 0.05).

Chapter 4: General Discussion

Varroa destructor are a type of pest that pose serious threat to honey bee colonies around the globe *(Rosenkranz et al., 2010)*. Due to stringent quarantine regulations, Australia was the only nation free of varroa mites until 2022; however, the mites have since been found there (Bourke et al., 2024). Varroa infestations do not exist in isolated areas like Antarctica and a few remote islands (Warner et al., 2024). In Canada, varroa mites, are leading causes of honey bee colony losses, especially during the winter season (Currie et al., 2010; vanEngelsdorp & Meixner, 2010). All growth phases of honey bee development are impacted by the parasitic varroa mite. These mites reproduce inside honey bee cells and they spread viruses like DWV-A and DWV-B both within and between colonies, making them a serious threat (Genersch & Aubert, 2010). In order to survive the winter, honey bees store proteins, of which vitellogenin is a significant component (G. V. Amdam et al., 2012; Otis et al., 2004). By puncturing the exocuticles of honey bees, the mites feed on their body reserves of fat body and hemolymph (Han et al., 2024). Since an infected colony can readily spread the mites to nearby colonies, controlling varroa mites throughout an entire apiary is imperative. The fall season is a crucial time for managing varroa mites because honey bees start reserving protein in the fall to survive through winters (Gatien & Currie, 2003; Mattila & Otis, 2007). The honey bees' survival into the next spring is directly impacted by their health during this stage.

The two primary wintering techniques used by beekeepers in Canada are indoor and outdoor (Kozak, 2008; Punko et al., 2021). During indoor wintering, colonies are stacked with inner covers on top of each hive and maintained at a regulated temperature of about 5°C (Punko et al., 2021; St. Clair et al., 2022). Colonies are protected from severe cold during outdoor wintering by being wrapped in insulating materials (Punko et al., 2021; St. Clair et al., 2022). The colonies may experience additional stress from protracted winters, which can last up to five months. Colonies need to be treated with acaricides to increase their chances of surviving if they have high varroa levels when they go into winter (Guzmán-Novoa et al., 2010). Controlling varroa mites is crucial for improving the health and immunity of honey bees because it also helps control viral infections (Dainat et al., 2012a; Francis et al., 2013).

This thesis explored the use of oxalic acid (OA) as treatment for varroa and viruses, with a focus on how treatment timing, application method and environment influence mite control and colony health. Through field and laboratory experiments, this work evaluated the outcome of oxalic on viral abundance in honey bees in the presence and absence of varroa. In Chapter 2, early fall trickle applications of 3.5% OA were tested in field colonies with brood, as well as in cage experiments without varroa mites to isolate virus level effects. The field study showed that a single 3.5% OA application temporarily increased mite mortality but had limited impact on overall mite abundance, colony strength, or protein levels. However, reductions in DWV-A and SBV were observed in surviving colonies in spring. Complementary cage experiments revealed mixed effects on virus levels, including reductions in BQCV and SBV with 2% or 3% OA or buffer treatments, but increases in DWV-B, suggesting virus specific responses to OA exposure. Chapter 3 focused on late fall 1g OA vaporization treatments, comparing different application intervals and frequencies in colonies exposed to varying initial mite loads and wintering conditions. The results demonstrated that four 1g OA applications were generally more effective than single 1g OA application, especially in colonies with low mite infestations, and this led to improved winter survival.

However, treatment efficacy was variable, likely due to ambient temperature , and mite suppression was inconsistent across trials.

Parsons, (2017) demonstrated that a 3% OA drizzle applied in early fall increased honey bee survival without significantly lowering varroa mite levels. However, (Parsons, (2017) study was limited by a small sample size, with only four treated and four control colonies, which may not have been sufficient to fully detect the effects of OA. In contrast, this study involved a larger sample of 20 treated colonies and 20 untreated colonies. In Chapter 2, 5 ml of a 3.5% OA solution was applied into each bee space using the trickle method on September 8, 2021. The results, measured by the Daily Mite Mortality Rate (DMMR), showed a significant increase in mite mortality two weeks after treatment in the oxalic-treated colonies compared to the controls. This contrasts with Parsons, (2017), which found no significant difference in mite levels, either in mean abundance or daily mite mortality, after OA treatment. Despite this higher mite mortality in oxalic treated colonies in current study, neither the mean varroa abundance nor the cluster score or colony weight showed any improvement in comparison to controls.

Chapter 3 described two experiments conducted in late fall 2020 and 2021, where 1g OA was applied in vapor form. During the fall of 2020, we tested both single and multiple (four treatments spaced 2-5 days apart) sublimation applications of 1g OA. For single applications, the treatment date was November 13, and for multiple applications, treatments were applied on November 13, 16, 18, and 23. The results showed that the single application on November 13 increased mite drop lasting until November 23, while the multiple applications produced a significantly longer effect, lasting until November 30 compared to controls. On November 30, these colonies were placed in wintering environments. Overall, all 1g OA treatments increased mite fall at different time points, with effects lasting from 1 to 2 weeks after treatment. Similar results were observed in fall 2021 experiment, but the sticky board mite drop was converted to DMMR for this experiment. In late fall 2021, we applied

sublimation 1g OA at 5-day intervals (from October 19 to November 3), and at 12-day intervals (from September 27 to November 2). The application of oxalic increased DMMR in comparison to controls, only within the colonies that had high mite levels in September. Specifically, the results showed that the 1g OA application at 5-day intervals showed high DMMR on November 9, and the 12-day intervals showed higher DMMR on November 12 in comparison to controls. On November 9, after replacing the sticky boards, the colonies were moved from Glenlea Research Station to campus on trucks. This movement of bees might have increased the effectiveness of OA by spreading it within the colony, leading to higher DMMR in treated colonies for the sticky boards collected on November 12. On November 19, all colonies were placed in a wintering environment.

In both the 2020 and 2021 experiments, colonies with multiple applications received a total of four applications. In both experiments, 1g of OA was applied using ProVap applicator. Comparing the intervals of the four applications for both vapor experiments, the 2020 experiment, with variable intervals of 2-5 days, demonstrated higher mite drop after each application, while the 2021 experiment, with treatments applied at a constant 5-day interval, showed high DMMR only after the last application. Additionally, the treatments applied at a constant 12-day interval showed high DMMR once, following the influence of the movement of colonies in trucks. This suggests that shorter intervals are more effective than longer intervals. However, the timing of the treatments likely played a role as well. In 2020, the treatments with a 2-4 day interval were applied between November 13 and November 23, while in 2021 those with a 5-day interval were applied between October 19 and November 3, and the 12-day interval treatments were applied from late September to early November (September 27–November 2). These differences suggest that treatments applied later in the fall are more effective than those applied earlier. The decrease in temperature during November likely contributed to this, as colder weather leads to reduced brood rearing, exposing mites and making them more vulnerable to the effects of OA treatment

(Mattila & Otis, 2007; Vetharaniam, 2012). This suggests that the best time to apply OA is later in the fall or just before winter, during the broodless period. Similar conclusions have been made in other studies, which report lower efficacy of OA when brood is present (Bubnič et al., 2024; Mutinelli et al., 1997; Rademacher & Harz, 2006). However, the difference between the two experiments could also be due to the methodology used, as in 2020 mite drop data was used, but in 2021, DMMR was used.

In this study, two approaches were used to assess *Varroa destructor* mortality through sticky board counts, reflecting differences in experimental design and colony mite levels between years. In the 2020 experiment, mite fall was assessed by counting the number of mites that accumulated on sticky boards between each treatment. This method was appropriate because colonies were initially balanced for mite levels, and the total number of mites remaining in colonies at the end of the experiment was also quantified.

In contrast, the 2021 experiment employed a DMMR approach, as described by Underwood & Currie (2005) to account for differences in initial mite infestations. DMMR is a more appropriate metric when colony mite loads are not equalized prior to treatment, as it normalizes mite fall over time and relative to baseline infestation levels. Without this adjustment, colonies with higher initial mite burdens may appear to have more effective treatments due to higher starting populations, rather than true treatment efficacy.

During the winters, the sticky board data revealed that the control colonies had more mites than the colonies treated with oxalic vapor in both years. This could be because of the natural fall of varroa mites. During the fall season, oxalic-treated colonies had higher mite mortality/fall, leaving very few mites in the colony to fall on the sticky boards in the following winter. On the other hand, control colonies had less mite fall/mortality during the fall season, which led to increased varroa fall during the following

winters. A similar pattern was observed in another study, where mite fall initially increased in treated colonies, and later, controls showed higher mite fall than treated colonies (Al Toufailia et al., 2015). On the contrary side, colonies that received multiple vapor oxalic treatments showed a significantly lower mean varroa mite abundance in the spring of 2021, compared to controls. Where as, there was no significant difference in the mean abundance of mites in spring 2022 for the control and treated colonies.

Parson's thesis recommended looking into how it affects viruses because OA may increase colony survival by lowering viral loads (Parsons, 2017). For both trickle and vapor fall season oxalic treatments,the suppression of DWV-A in spring of next year was observed, and SBV was also suppressed in spring following fall season oxalic trickle treatment. In September 2021, we used the trickle method to carry out the field experiment described in Chapter 2. There was no long term effect of fall season trickle oxalic treatment on varroa mites in spring. In comparison to controls, 3.5% trickle OA had a long-term effect, lowering DWV-A and SBV levels by April 2022. Similarly, Chapter 3 describes how the application of 1g vaporized OA in November 2020 affected honey bees in the spring 2021. In spring 2021, varroa was suppressed more in colonies that received multiple applications than in colonies that received single application in fall season. On the other hand, DWV-A was suppressed more in colonies that received a single application than in colonies that received four. Although the exact mechanisms are still unknown, these results suggest that while excessive use of OA can increase virus levels, limited use may gradually suppress the virus. Similar results were observed in treated (tau-flauvalinate) colonies that had higher DWV virus than controls (Locke et al., 2012).

In the 2020-2021 experiment, mite drop on sticky boards was higher in indoor colonies in comparison to outdoor colonies in December and January. The DWV-A level was higher in outdoor colonies. Even after higher stress factors, for the 2020-2021 experiment, the survival of colonies was

higher for outdoor wintering for experiment one in Chapter 3. In this experiment, 53% of the indoor and 80% of the outdoor colonies survived.

Similar to the previous year, in the 2021-2022 vapor experiment, the DWV viral load was higher in outdoor colonies but with the DWV-B variant. This shows higher stress levels for colonies wintering outdoors in comparison to indoor colonies. For the 2021-2022 vapor experiment, colonies wintered indoors with low mite load showed lower weights than outdoor wintered colonies. Likely because of less stress by viruses and mites. In this experiment, 26% of indoor colonies survived, while 63% of outdoor colonies survived.

Spring Virus levels varied with levels of initial varroa mite infestation before wintering. In Chapter 2, BQCV viral titers in April were lower in colonies with low initial varroa levels. (Doublet et al., 2024; Manley et al., 2020) observed that BQCV had higher prevalence and viral load in colonies with higher varroa, which indicates that BQCV might be transmitted by varroa. But for DWV-A, viral titers in April were higher in colonies with low initial varroa levels. This is unusual as varroa is a vector of the DWV-A virus. This could be explained because if the mean abundance of varroa mites and the levels of DWV-A increased with time and the colonies with high stress of pathogens died the levels in surviving colonies would be low.

In Chapter 3, DWV-B viral titers were lower in November 2022 for the colonies with low initial varroa loads in September 2021. (McAfee et al., 2025), did not find any co-relation between varroa and DWV-B in one of their study years. Additionally, we noticed that DWV-B viral loads were impacted by interactions between oxalic treatment and varroa levels. Within colonies treated with 5-day and 12-day 1g OA treatments, viruses were reduced in colonies with low varroa levels, this trend was not observed for control colonies.

Virus levels varied with time from September 2021 to April 2022 for both experiments. In the 3.5% oxalic trickle method, DWV-A and DWV-B gradually increased, while BQCV and SBV mostly declined. However, BQCV showed some ups and downs, dropping at first but later rising again. In the 1g vaporized OA treatment, DWV-A and DWV-B followed a similar pattern, increasing at first but then dropping by April. BQCV also declined in the early months but later rose again. SBV levels fell early and stayed low, while IAPV kept rising steadily. Desai & Currie (2016a), observed that SBV levels were higher in spring in comparison to fall season.

Looking at both treatments together, DWV-A and DWV-B behaved similarly in both, increasing early on, but in the vaporized treatment, they eventually declined. BQCV followed the same fluctuating pattern in both cases. SBV stayed low in both treatments. The biggest difference was in IAPV, which only showed continuous growth in the vaporized treatment. These findings suggest that while some viruses react similarly no matter the treatment, others respond differently based on how OA is applied.

Chapter 2 has two cage experiments, which were conducted to observe the direct impact of oxalic on honey bee viruses in the absence of varroa mites. Australian honey bees imported in 2021 were used for this experiment to ensure varroa-free bees. Australian colonies were kept secluded from surrounding honey bees to prevent any varroa infestation. The 3.5% trickle OA treatment method was replicated in caged bees by pipetting 2% or 3% OA on each bee. Some cages were left with natural virus levels. However, to increase the level of viral infection, some cages were orally fed with DWV inoculum. The inoculum used for experiments was DWV-A and DWV-B. For extraction of these viruses, extraction buffer (0.1 M ascorbic acid and 0.5 M potassium phosphate), was used.

In the cage experiments, we observed reduction of BQCV in 3% OA treated cages, but it was influenced by effect of buffer. Results showed that buffer reduced buffer reduced levels of BQCV and

SBV. This suggests that components of the buffer may have antiviral properties, which could confound the interpretation of virus suppression attributed solely to OA. In one of the cage experiments 2% oxalic increased levels of DWV-B in naturally infected honey bees.

Another factor that affects honey bee health is protein levels. In second experiment of Chapter 3, interactions between the initial varroa mite levels and the 12-day oxalic treatment had an impact on protein levels. Protein levels were higher in colonies with lower initial varroa loads, confirming the theory that varroa mites reduce honey bee protein stores, which affects survival (Bowen-Walker & Gunn, 2001). This reduction of protein levels was only observed in 12-day but not in 5-day treated colonies. It indicates protection provided by a 5-day oxalic treatment, which allowed honey bees to have similar protein levels in both high and low varroa-infested colonies. However, it is possible the no difference was observed between protein levels in colonies with high or low varroa levels because the treatment was applied after the formation of honey bees. In Chapter 3, for Experiment 2, protein levels increased from September to November, but the increase was not statistically significant. This increase in protein levels indicates the transition of summer bees to winter bees, that accumulate proteins to survive throughout the winter. However in Chapter 2, protein levels were highest in September, reduced in November, and decreased further in spring. This could be explained by the emergence of winter bees earlier in September in that year. Both of these experiments were conducted in fall season 2021. In spring 2022, both experiments had very low levels of proteins, it could be because of higher levels of varroa mites in spring. For both experiments in 2021-2022, mean abundance of varroa mites increased over time regardless of treatments.

We saw 1g OA vaporization affected cluster size before wintering in November (Chapter 3 experiment 1). Multiple applications of oxalic at variable intervals showed higher mite mortality than other treatments. This led to less mortality of bees and bigger cluster sizes in November. On November 26th, the cluster sizes of the colonies treated four times at 2-5 day intervals were larger than those of the

single-treatment and control colonies. There was no effect on colony weight in November. On the other hand, colony weight in spring was affected by oxalic application, as colonies treated four times with 2-5 day intervals showed lower weights than the single treatment and control colonies. It could be because, in November these colonies had bigger population of honey bees which consumed more syrup, leading to lower weight in spring. However, there was no effect of oxalic on cluster size in spring. For both oxalic vapor and trickle experiments conducted in 2021-22, overall cluster size and colony weight for 2021 was reduced from fall to spring season. This is usually expected due to normal honey bee mortality because of higher levels of mites in spring 2022. In spite of low oxalic efficacy, survival of honey bees was affected by 1g OA vapor method conducted in 2021-2022.

The survival of colonies in the low initial varroa mite group was greatly impacted by oxalic in Chapter 3. Colonies treated at 5-day intervals (9 colonies) survived significantly better than those treated at 12-day intervals (4 colonies) or left untreated (3 colonies). In total, the spring survival rates for the controls were 7 colonies, the 5-day treatment was 11, and the 12-day treatment was 7. In other experiments, oxalic had no effect on survival. In the first experiment (conducted in 2020) described in Chapter 3, 66% of the colonies made it through the spring. There were 14 colonies that survived in the control group, 12 in the single treatment group, and 14 in the four-time oxalic-treated colonies at variable intervals of 2–5 days. In Chapter 2, 12.5% of the colonies survived in spring after a single early fall season oxalic trickle treatment; two oxalic-treated colonies and three control colonies survived.

Conclusion

This study highlights how managing varroa mites is key to keeping honey bee colonies healthy, especially during Canada's long winters. *Varroa destructor* weakens bees by feeding on their body reserves and spreading dangerous viruses like DWV-A and DWV-B. Our research tested two OA

treatments - 3.5 % trickle and 1g vaporization to see how well they controlled mites, reduced viruses, and supported colony health.

We found that OA helped kill mites, but its effectiveness depended on how and when it was applied. The trickle method, used in early fall season, increased mite drop but didn't significantly lower mite levels in the long run. Vaporized OA, applied in late fall season, worked better, especially when treatments were done closer together (every 2-4 days instead of 5-12 days). Treatments in November were more effective than those done earlier in the fall season.

Oxalic also affected virus levels. Colonies treated with OA had lower DWV-A and SBV levels by spring, but using it too often seemed to increase virus levels instead of reducing them. Our cage experiments showed no direct effect on viruses, suggesting 2% or 3% OA helps control viruses mainly by reducing mite infestations rather than acting on the viruses themselves.

Colony health and survival were also impacted. Colonies with fewer mites had higher protein levels, and those treated multiple times with vaporized OA had larger winter clusters. Colonies that received oxalic treatments every 5 days had the highest survival rates, showing that proper treatment timing matters.

Wintering conditions also played a role. Colonies kept outdoors had higher mite drops and larger clusters compared to those wintered indoors. DWV-A was more common outdoors, while IAPV was higher in indoor colonies.

Overall, our study shows that OA is a valuable tool for varroa control, but how and when it's used makes a big difference. Treating colonies in November with vaporized OA at short intervals (every 2-4 days) provides the best mite control and virus suppression, improving colony survival through winter.

Beekeepers need to carefully time and space their treatments to get the best results and keep their bees healthy.

Future research

Future studies could be conducted to test the direct effect of OA on honey bees in the absence of varroa mites in laboratory conditions. Future studies could benefit from using a greater number of replicates involving naturally infected honey bees, without addition of virus inoculation, to eliminate the confounding effects of buffer solutions. Additionally, separate experiments should be conducted using buffers alo ne to further investigate their influence. This would allow for a clearer understanding of the individual roles of oxalic OA and the buffer in virus suppression.

The mechanism by which OA controls varroa mites remains unclear. (Parsons, 2017) suggested that OA may function as an antifeedant. To test this hypothesis, a cage experiment using fluorescent biostain was carried out from 8 December to 19 December 2022. The experiment included 25 plexiglass cages, each containing 60 Australian honey bees. Honey bees in 20 cages were fed a fluorescent biostain for 5 days. The biostain was prepared by mixing 0.05 g of Nile red, 1 ml of sunflower oil, 1 g of soy lecithin (emulsifier), 10 g of honey (stabilizer for emulsifier), and 60 g of sucrose in 200 ml of water (Ramsey et al., 2019). The remaining 5 cages were fed honey sucrose syrup (5 g honey, 30 g sucrose, and 100 ml water). These 25 cages were kept in the incubator for 5 days.

After 5 days, 10 bees were randomly selected from each cage and placed individually in 10 modified Petri dishes. The Petri dishes were modified by creating two holes in the lid, one sealed with mesh and the other with a cork. A layer of parafilm was wrapped around the lateral side to prevent mites from escaping. Petri dishes from the control cages (not fed on biostain) were used as controls. Of the 20 cages that were fed on biostain, Petri dishes from 10 cages were treated with OA, and the remaining were

left untreated. OA was applied by pipetting 4.1 µl of 3% OA in 1:1 sugar syrup per honey bee. Two varroa mites were placed on each bee in the Petri dish. A sugar cube was added for bee feeding, and water was supplied. These Petri dishes were kept in the incubator for 24 hours.

After 24 hours, the honey bees and mites were anesthetized with CO_2 to remove mites. The mites were rinsed with 70% ethanol to remove any remaining biostain. Each mite was placed in a tube with 30% hydrogen peroxide for 5 days to quench autofluorescence. The mites were then observed using a BioTek Cytation 5 Cell Imaging Multimode Reader to assess fluorescence levels. Mites were placed in 96-well plates and observed using an RFP imaging cube in a multimode Bio-Rad reader, and fluorophore data were recorded. Preliminary results did not show a difference in fluorophore between the mites that fed on biostain-fed bees and those that fed on control bees. These findings suggest that the experiment may not have successfully captured differences in feeding behavior, possibly due to insufficient uptake or detection of the dye. Future experiments could be conducted with improvements in detection sensitivity and dye incorporation to better test the hypothesis that OA functions as an antifeedant.

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