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ORIGINAL RESEARCH ARTICLE

Effects of treatments with Apivar® and Thymovar® on *V. destructor* populations, virus infections and indoor winter survival of Canadian honey bee (*Apis mellifera* L.) colonies

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Efficacies of two miticides, Apivar® and Thymovar®, were evaluated as a fall treatment against *V. destructor*. The effect of treatment with miticides was further evaluated by monitoring both viral load and rate of indoor overwintering survival of colonies of European honey bees (*Apis mellifera* L.) in the vicinity of Saskatoon, Saskatchewan, Canada. Forty-five colonies were randomly assigned to three treatment groups with 15 hives per group: Group 1; 2 strips of Thymovar® (thymol); Group 2; 2 strips of Apivar® (Amitraz); and Group 3; no treatment (control). Significant decreases in the rates of colony infestation (Mites per hundred bees, MPHB) by *V. destructor* were observed ($p < 0.05$) between colonies of bees treated with Apivar® in October 2013 when compared to control colonies. Efficacy of Apivar® and Thymovar® against *V. destructor* after treatment for 22 days were 76.5 and 26.7%, respectively. After 22 days, concentrations of the two miticides in bees were 15.4 ng amitraz/g wet mass (wm) and 64,800 ng thymol/g wm. There were no significant differences ($p > 0.05$) in the percentage of colonies infected by deformed wing virus (DWV) and Israeli acute paralysis virus (IAPV) either before or after treatment with Apivar® or Thymovar® in October 2013 and 7 months post treatment in April 2014. Only the Apivar® treatment group showed IAPV infections in April 2014. The group treated with Apivar® exhibited a better overwintering rate of survival (93%), than hives treated with Thymovar® (67%). These results suggest volatile miticides like Thymovar® should be avoided in geographical areas with colder fall temperatures.

Efectos de los tratamientos con Apivar® y Thymovar® sobre las poblaciones de *V. destructor*, infecciones de virus y supervivencia interior durante el invierno las colonias de abejas de la miel (*Apis mellifera* L.) canadiense.

Se evaluó la eficacia de dos acaricidas, Apivar® y Thymovar®, como tratamiento de caída contra *V. destructor*. El efecto del tratamiento con acaricidas se evaluó mediante el control tanto de la carga viral como la tasa de supervivencia de hibernación interior de las colonias de abejas europeas (*Apis mellifera* L.) en las proximidades de Saskatoon, Saskatchewan, Canadá. Se asignaron cuarenta y cinco colonias aleatoriamente a tres grupos de tratamiento con 15 colmenas por grupo: Grupo 1; 2 tiras de Thymovar® (timol); Grupo 2; 2 tiras de Apivar® (Amitraz); y el Grupo 3; ningún tratamiento (control). Se observaron disminuciones significativas en los índices de infestación de colonias (ácaros por cien abejas, APCA) de *V. destructor* (P <0,05) entre las colonias de abejas tratadas con Apivar® en octubre de 2013, en comparación con las colonias de control. La eficacia de la Apivar® y Thymovar® contra *V. destructor* después del tratamiento durante 22 días fueron 76,5% y 26,7%, respectivamente. Después de 22 días las concentraciones de los dos acaricidas en abejas fueron 15,4 ng amitraz / g de peso húmedo (PH) y timol 64.800 ng / g PH. No hubo diferencias significativas (P> 0,05) en el porcentaje de colonias infectadas por el virus de alas deformadas (DWV por sus siglas en inglés) y el virus israelí de la parálisis aguda (IAPV por sus siglas en inglés) ya sea antes o después del tratamiento con Apivar® o Thymovar® en octubre de 2013 y 7 meses después de la tratamiento en abril de 2014. Sólo el grupo tratado con Apivar® mostró infecciones con IAPV en abril de 2014. El grupo tratado con Apivar® exhibió una mejor tasa de supervivencia después de la hibernación (93%), que las colmenas tratadas con Thymovar® (67%). Estos resultados sugieren que acaricidas volátiles como Thymovar®, se deben evitar en las zonas geográficas con temperaturas más frías de otoño.

Keywords: miticide residues; deformed wing virus (DWV); Israeli acute paralysis virus (IAPV); colony failure; survival

Introduction

Declines in populations of honey bees are of global concern to agriculture, because each year the European honey bee (*Apis mellifera*) adds approximately $40 billion to the world economy (Fairbrother et al. 2014; Klein et al., 2007). Although causes of increased rates of failure of colonies are still unclear, results of some studies have suggested that extensive use of insecticides might be a responsible co-factor for failures of colonies (Al Naggar, Codling, et al., 2015a, Al Naggar, Vogt, et al., 2015b; Mullin et al., 2010). The major cause of colony loss is, however, thought to be due to parasitism by the mite

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Varroa destructor and associated pathogens (Martin et al., 2012, Ryabov et al. 2014). Evidence for exposure to imidacloprid and increased varroa mite levels have been reported (Dively, Embrey, Kamel, Hawthorne, & Pettis, 2015), indicating colony health may also be dependent on interactions with environmental pesticides.

Controlling populations of *V. destructor* in colonies of European honey bees is challenging because of cost, potential for contaminating products of treated hives, different climatic conditions, and resistances of the mites to synthetic acaricides (Floris et al., 2001; Le Conte, Ellis, & Ritter, 2010; Mozes-koch et al., 2000; Spreafico, Eördegh, Bernardinelli, & Colombo, 2001). For these reasons, beekeepers have been trying alternative treatments that incorporate essential oils and organic acids (Gregorc & Poklukar, 2003; Melathopoulos & Gates, 2003).

Thymovar® is a trade name for a thymol product that is sold commercially to treat bee colonies for infestation with mites. Thymol is widely used in honey bee colonies as a treatment against both *V. destructor* (Fassbinder, Grodnitzky, & Coats, 2002; Imdorf, Bogdanov, Ibanez Ochoa, & Calderone, 1999; Lindberg, Melathopoulos, & Winston, 2000) and tracheal mites, *Acarapis woodi* (Rennie), (Ellis & Baxendale, 1997). Thymol has been shown to act as a positive allosteric modulator of GABAA *in vitro* (García, Bujons, Vale, & Suñol, 2005). Apivar® is a commercial product that contains technical grade active ingredient amitraz. Amitraz is a nonsystemic, formamidine contact acaricide used to kill ectoparasites (Avarez-Ventur, 2011; Corta, Bakkali, Berrueta, Gallo, & Vicente, 1999). It is thought to act on the nervous system (Dudai, Buxbaum, Corfas, & Ofarim, 1987; Evans & Gee, 1980) leading to over-excitation and consequently paralysis and death in arthropods. Apivar® is lipophilic and can contaminate beeswax, but it rapidly degrades through a series of intermediate compounds to form another environmentally stable, compound 2,4-dimethylaniline (2,4-DMA) (Bogdanov, Imdorf, Kilchenman, & Fluri, 1998; Smodiš Škerl, Kmeel, & Gregorc, 2010) that can also be toxic to insects. While it was thought to be relatively nontoxic to bees (Briggs, 1992), misuse and concerns about effects of amitraz on humans have made this chemical illegal for use on honey bee colonies. However, Health Canada's Pest Management Regulatory Agency (PMRA) has granted a conditional registration for the sale and use of amitraz technical material and Apivar® Strips to control *V. destructor* in honey bee colonies. (http://www.hc-sc.gc.ca/cps-spc/pubs/pest/_decisions/erc2013-04/index-eng.php).

The relationship between *V. destructor* and viruses in honey bees has been well documented in both laboratory and field studies (Berthoud, Imdorf, Haueter, Radloff, & Neumann, 2010; Carreck, Ball, & Martin, 2010; Todd, De Miranda, & Ball, 2007) and the outcomes of virus infections and rates of increase in populations of *V. destructor* and transmission dynamics have been modeled (Martin, 2001; Sumpter & Martin, 2004). The aim of this study was to evaluate and compare the acaricidal efficacy of a short-term Apivar® treatment and a standard Thymovar® treatment during fall for controlling *V. destructor* in colonies of the European honey bee in Saskatchewan, Canada. In addition, residues of miticides were measured in worker bees before and after treatments. The possible effects of these miticide treatments on deformed wing virus (DWV), Israeli acute paralysis virus (IAPV), and Kashmir bee virus (KBV) infections in adult bees, and indoor winter colony survival were also investigated.

Materials and methods

Experimental design

The experimental design for the study utilized a total of 45 colonies of the European honey bee (*A. mellifera*) in apiaries operated by Meadow Ridge Enterprises LTD Saskatchewan, Canada (52°11′ N, 106°63′ W) between 14 September and 05 October 2013. Colonies were re-evaluated seven months post-treatment, at the end of April 2014. Two story colonies were normalized for strength, based on the number of standard, deep Langstroth frames of bees (14 to 16). These colonies all had top entrances (2 inches wide and 0.5 inches high), with a 0.75 inch high bottom entrance across the length of the bottom brood chamber. On 14 September 2013, colonies were randomly assigned to 3 groups with 15 colonies per treatment: Group 1–2 strips of Thymovar®; Group 2: 2 strips of Apivar®; and Group 3: no treatment (control). Apivar® strips were removed after 22 days (2.5–3 life cycles of *V. destructor* in bee brood). Thymovar® strips remained in the colonies to be chewed and removed by the bees. Colonies were moved indoors on 30 October 2013 for wintering, and moved outside on 11 April 2014 and evaluated on 30 April 2014. The ambient indoor temperature was maintained at 4 ± 3 °C between October and 11 April 2014.

Data and sample collection

An initial set of measurements and samples were taken from colonies prior to treatments. Infestation with *V. destructor* (Mites per hundred bees, MPHB) was determined by washing 150 or more bees with methanol to dislodge mites (Fries, Aarhus, Hansen, & Korpela, 1991). Another sample of 100 bees from the same brood frame was collected and placed immediately on dry ice, and then stored at −80 °C until processing for determination of infection with viruses and quantification of miticides. Twenty-two (22) days and 7 months after initiating exposures to miticides, samples of adult bees were collected to determine phoretic *V. destructor* infestations *(MPHB),* presence or absence of viruses and concentrations of miticides. All colonies were evaluated on 30 April 2015 for food reserves, strength, queen status, and overall colony health. A surviving colony was scored as one with brood and three or more frames of bees with the queen.

Identification of viruses in adult bees

RNA was isolated from composite samples of 10 adult bees, randomly collected from each colony, by use of previously described methods (Robertson et al., 2014). A probability of finding an infestation of 10% or more with a sample of 10 bees would be expected to score positive. Samples of bees were screened for DWV, IAPV, and KBV in control or colonies treated with one of the two miticides, by use of reverse transcription PCR before and after the designated treatment. DWV and KBV were detected using the forward and reverse primers CAGTAGCTTGGGCGATTGTT, AGCTTCTG-GAACGGCAGATA, and GATGAACGTCGACC-TATTGA, TGTGGGTTGGCTATGAGTCA, respectively (Cox-Foster et al., 2007) and IAPV was detected using primers GCGGAGAATATAAGGCTCAG (forward) and CTTGCAAGATAAGAAAGGGGG (reverse) (Di Prisco et al., 2011). Presence of a single PCR product (after 30 cycles) of the expected size was confirmed by use of 2% agarose gels (Invitrogen, Burlington, ON, Canada) according to previously described methods (Locke, Forsgren, Fries, & De Miranda, 2012).

Quantification of miticides

Concentrations of Apivar® and its transformation products (2,4 Dimethylaniline (DMA) and 2,4 Dimethylphenyl formamide (DMPF) and Thymovar® were measured in samples of bees before (14 September, 2013) and after treatment with Apivar® or Thymovar®. Two composite samples from each treatment group (n = 15) were made and then 3 g of each sample analyzed by use of method AOAC 2007.01 of the Association of Official Analytical Chemists (AOAC) (Lehotay et al., 2007). This Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) method uses a single-step buffered acetonitrile (MeCN) extraction and salting out liquid–liquid partitioning from the water in the sample with $MgSO_4$. Dispersive-solid-phase extraction (dispersive-SPE) cleanup is done to remove organic acids, excess water, and other components with a combination of primary secondary amine (PSA) sorbent and $MgSO_4$. Extracts were analyzed by mass spectrometry (MS) after gas chromatographic (GC) separation at National Science Laboratories (Gastonia, NC, USA).

Efficacy of miticides against V. destructor

Efficacy of the applied miticides against infection with the mite *V. destructor* was calculated by use of the Henderson-Tilton formula (Henderson & Tilton, 1955) (Equation (1)).

$$\text{Corrected}\% = \left(1 - \frac{n \text{ in } Co \text{ beforetreatment} * n \text{ in } T \text{ after treatment}}{n \text{ in } Co \text{ after treatment} * n \text{ in } T \text{ before treatment}}\right) \times 100 \quad (1)$$

where n = Insect population-colony, T = treated, Co = control.

Statistical analysis

Normality was confirmed by the Kolmogorov–Smirnov test and homogeneity of variance was confirmed by use of Levine's test. Transformation of data was done when required to meet these assumptions of parametric statistics. Differences among rates (MPHB) of infestation with *V. destructor* in the three treatment groups after treatment in October 2013 and April 2014 were firstly assessed by Two-way analysis of variance (ANOVA) (treatment x time) however there was no interaction, so One-way analysis of variance (ANOVA) followed by a Tukey's *post hoc* test were used. Infection with DWV, IAPV, and KBV in the three treatment groups were scored as a positive or negative and analyzed using the Chi Square (X^2) test. For all analyses the level of Type I error was set as $p < 0.05$.

Results

At the beginning of the experiment, mean rates of infestation with phoretic *V. destructor* (MPHB) of bees of all experimental colonies were 3.2 MPHB. There were no significant differences ($p > 0.05$) in rates of infestation with *V. destructor* MPHB among replicates assigned to experimental groups prior to treatment. Twenty-two days post treatment (October 2013) mean rates of infestation of bees with *V. destructor* (MPHB) were 1.5, 4.5, and 6.3 in colonies treated with Apivar®, Thymovar® and in controls that were not treated with a miticide, respectively. Seven months post treatment (April 2014) mean rates of infestation with *V. destructor* were 2.9, 4.3, and 4.9 MPHB, respectively, in colonies treated in the fall of 2013 with Apivar®, Thymovar®, and Control, respectively (Figure 1). A significant decrease was observed (F = 3.89, df = 2, $p = 0.02$) in rates of infection with *V. destructor* among bee colonies treated with Apivar® only in October 2013 when compared to control colonies. The efficacy of Apivar® or Thymovar® against *V. destructor* after treatment for

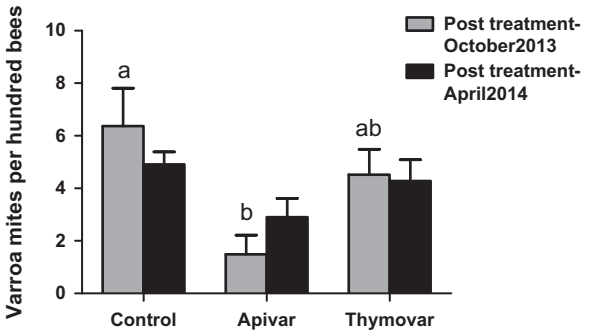

Figure 1. Mean (± SEM) (n = 15) rate of infestation of bees with *V. destructor* (Mites per hundred bees, MPHB) after treatment with Apivar and Thymovar in October 2013 and April 2014.
Note: Means with different letters are significantly different (one-way ANOVA with tukey *post hoc* test, $p < 0.05$).

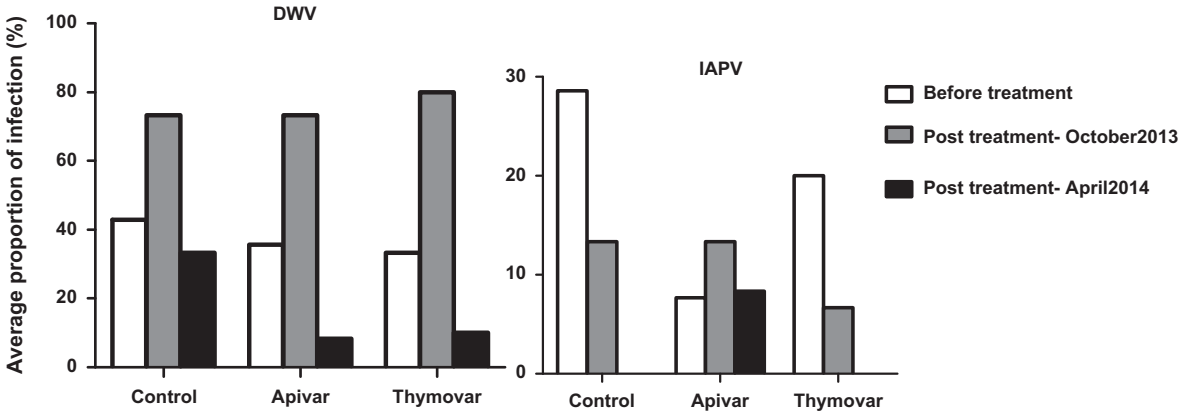

Figure 2. Mean proportion of colonies infected with DWV and IAPV before and after treatment with Apivar and Thymovar in October 2013 and April 2014.

22 days on 05 October 2013 was 76.5 and 26.7%, respectively.

There were no significant differences in rates of colony infections with DWV before treatment ($X^2 = 0.30$, $p = 0.86$) and after treatment with Apivar® in October 2013 ($X^2 = 0.24$, $p = 0.88$) or April 2014 ($X^2 = 3.15$, $p = 0.20$). Rates of colony infections with IAPV were not significantly different before treatment ($X^2 = 2.15$, $p = 0.34$) and after treatment with Thymovar® in October 2013 ($X^2 = 0.45$, $p = 0.79$) or April 2014 ($X^2 = 1.88$, $p = 0.38$). Relative to pretreatment values, an increase in the mean rate of composite samples of colonies infected with DWV was observed on October 2013 with 73.3% for colonies treated with Apivar® and untreated (control) and 80% for Thymovar® treated colonies. In contrast, a decrease in the mean rate of composite adult bee samples from colonies infected with DWV were 8.3% and 10% for colonies treated with Apivar® or Thymovar®, respectively, and 33.3% for control colonies in April 2014 (Figure 2).

Mean rate of composite samples of colonies infected with IAPV before treatment with miticides were 7.7, 20, and 28.5%, for colonies treated with Apivar®, Thymovar®, or control colonies, respectively. After treatment on 05 October 2013 Control and Apivar® treated colonies were infected with a mean rate of infection with IAPV of 13.3%, while 6.7% of colonies treated with Thymovar® were infected. In April 2014, only Apivar® treated colonies (8.3%) showed IVAP infections. KBV was not detected before or after any of the treatments (See online supplementary information).

Colonies treated with Apivar® exhibited the greatest rate of overwintering survival (93%), while the colonies treated with Thymovar® exhibited the poorest rate of survival (67%). The control treatment group had 80% winter survival. However, there were no significant differences ($p > 0.05$) in overwintering rates.

Concentrations of miticides were measured before (14 September 2013) and after treatment (5 October 2013) with Apivar® or Thymovar®. Concentrations of amitraz were detected only after treatment with Apivar®, with a mean of 15.4 ng/g, wet mass (wm). No metabolites of amitraz were detected in any samples. Thymol was detected in all samples with the greatest concentration being 64,800 ng/g, wm in samples of bees after treatment with Thymovar® (Table 1).

Discussion

Efficacy of control of *V. destructor by* Thymovar® was 26.7%, compared to Apivar® which had an efficacy of 76.5%. The efficacy of Thymovar® to control *V. destructor* observed in the current study was not consistent with that reported previously in which miticide efficacies for Thymovar® ranged from 68.7 to 99% (Baggio, Arculeo, Nanetti, Marinelli, & Mutinelli, 2004; Bogdanov et al., 1998; Bollhalder, 1999; Higes, Suarez, & Llorente, 1996; Imdorf, Carriere, Klichenmann, Bogdanov, & Fluri,

Table 1. Concentrations of miticides (ng/g, wm) in bees before and after treatment with Apivar and Thymovar October 2013.

	Concentration (ng/g, wm)				
		After treatment			
Compound	Before treatment	Control	Apivar	Thymovar	LOD (ng/g)
Amitraz	ND	ND	15.4	ND	4
2,4 DMA	ND	ND	ND	ND	50
2,4 -DMPF	ND	ND	ND	ND	4
Thymol	408	245	1,660	64,800	50

Note: LOD = Limit of detection.

2003).The efficacy observed in the current study was, however, similar to that observed by Gregorc and Planinc (2012). Those authors reported an efficacy for Thymovar® of 14.35 ± 10.71%. This discrepancy is probably due to different climatic conditions and geographic locations, particularly temperature and hive management systems (Trouiller & Watkins, 2001). Moreover, there were differences among studies in the formulations, doses, and time of application of acaricides, which could also affect efficacy. Thymovar® showed treatment efficacies similar to Apivar® in both spring and fall treatments, but Thymovar® showed significant decreases in brood production in spring applications (Vandervalk, Nasr, & Dosdall, 2014). Hive ventilation may play a role in treatment efficacies and brood production. We used top covers with 2 inch by 1/2 inch opening which would allow more air flow. These parameters need further investigation.

In the current study, treatment with Apivar® resulted in an efficacy of 76.5%, which is in agreement with results of previous studies, during which an efficacy of 86% was observed (Avarez-Ventur, 2011). A relatively short exposure of 22 days (2.5 to 3 life cycles of *V. destructor* in bee brood) was used with 2 Apivar® strips, where the recommended treatment is 42 days. Treatments during fall in temperate climates can be problematic, because of low temperatures and clustering behavior of bees which reduce exposure to the miticides. The mean maximum and minimum temperatures during the treatment period were 18C and 5C, respectively (Online supplementary information Figure S1). Colonies need time to reseal after opening in preparation for winter, and strips must be removed to avoid long exposures, which can promote development of resistance by *V. destructor*. This shorter duration treatment, with two strips resulted in good efficacy, which may have also been affected by the lack of drone brood in colonies during fall. Apiaries used in this study had not been previously treated with Apivar®. Meadow Ridge apiaries use stock selected for resistance to mites and oxalic liquid and thymol are applied in spring to control *V. destructor*. Treatments with miticides in fall are avoided, because of added colony stresses imposed by the miticides before wintering.

Resistance of mites to Apivar® has been reported in Minnesota and other areas in the United States of America (Elzen, Baxter, Spivak, & Wilson, 2000; Milani, 1999). Also, mites have developed resistance to amitraz where it has been used for many years in Europe (Oliver, 2007).On the basis of the results of this study, it can be concluded that mites in this Saskatchewan apiary were not resistant to Apivar®.

DWV has become the most prevalent virus associated with infestations of honey bees by *V. destructor* (De Miranda & Genersch, 2010; Genersch & Aubert, 2010). Thus, removal of mites using an acaricide treatment should result in a progressive reduction in DWV infections (Martin, Ball, & Carreck, 2010). In contrast, in this study the mean proportion of colonies infected with DWV after treatment with Apivar® or untreated controls on 5 October 2013 was 73% compared to 80% for Thymovar® (Figure 2 and online supplementary information Tables S1 to S3). The mean rate of infection of colonies with DWV did not decrease after treatment with Apivar® even though infestations with *V. destructor* decreased from 3.1 to 1.5 MPHB.

A number of factors could be causing this temporary increase in infections with DWV. The miticides used kill primarily phoretic phase mites and not mites already reproducing in the brood. However, miticide treatments reduce the number of phoretic mites resulting in lower rates of brood re-infestation. Adult bees with phoretic mites previously attached would remain infected with viruses until death. Larger numbers of bees infected with virus would be expected to die before spring because of lesser life span (Dainat et al., 2012), but they may not have died before the first post-treatment assays were performed. This could explain the difference between rates of infection with viruses 22 day post-treatment and those observed in April. Other factors, such as virus contamination of brood food, virulence of the DWV, spread of infection from bee to bee (horizontal) or vertically through infected queens could also play roles in spreading virus infections (De Miranda, Gauthier, Ribiere, & Chen, 2012; Locke et al., 2012). Another possibility that needs to be investigated is the direct effects of the miticides (Apivar® and Thymovar®) on immune responses of the host to infection by virus. Recent observations following treatment with Apistan also showed an increase in rates of infection with DWV (http://www.livescience.com/18139-honey bee-mite-virus.html) after treatment. Treatments with Apivar® or Thymovar® have a random rather than progressive influence on rate of infection with IAPV.

Together these observations suggest further investigation on the effects of miticides on bee health is warranted. In the absence of *V. destructor* infestations, various miticides can have effects on the expression of honey bee genes involved in detoxification, immune, and other metabolic and developmental pathways (Boncristiani et al., 2012). Thymol, as the Apiguard formulation was shown to alter gene expression in a number of the above pathways.

Thymovar® residues detected in adult bees before treatment with Thymovar® are of interest. Since colonies were treated with Thymovar® (two strips, three weeks, repeated once) in spring (May) of 2013, bees contained 408 ng thymol/g, wm (Table 1) until mid-September. This is of interest since the population would have turned over since the application in the spring, but traces of thymol were still detectable in worker bees. This suggests thymol can persist in wax and hive products in colony brood nests, to which bees of all life stages are exposed. This persistence is of concern and requires further investigation. Concentrations of fluvalinate, coumaphos, and amitraz metabolites (DMA and DMPF) were detected in samples of wax and

honey bees from North American sources (Mullin et al., 2010), but no tests for thymol or amitraz were done. To our knowledge, this is the first study to report thymol and amitraz levels in adult honey bees after a 22-day controlled treatment for *V. destructor*.

Apivar® exhibited excellent efficacy (76.5%) against *V. destructor* following a short fall treatment (22 days) in a temperate climate. Thymovar® showed an efficacy of 26.7% suggesting this type of treatment should be avoided in late fall treatments in geographical areas with cold fall temperatures similar to the Canadian prairies.

Supplementary material

The supplementary material for this paper is available online at http://dx.doi.org.10.1080/00218839.2016.1186917.

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