diversity

MDPI

Article

Control of *Varroa destructor* Mite Infestations at Experimental Apiaries Situated in Croatia

Ivana Tlak Gajger [1,*], Lidija Svečnjak [2], Dragan Bubalo [2] and Tomislav Žorat [3]

1 Department for Biology and Pathology of Fish and Bees, Faculty of Veterinary Medicine, University of Zagreb, 10000 Zagreb, Croatia
2 Department of Fisheries, Apiculture, Wildlife Management and Special Zoology, Faculty of Agriculture, University of Zagreb, 10000 Zagreb, Croatia; lsvecnjak@agr.hr (L.S.); dbubalo@agr.hr (D.B.)
3 Veterinary ambulance Obrovac d.d., 23450 Obrovac, Croatia; tomislav.zorat@gmail.com
* Correspondence: ivana.tlak@vef.hr; Tel.: +385-91-2390-041

Received: 5 December 2019; Accepted: 22 December 2019; Published: 25 December 2019

Abstract: Experimental varroacidal treatments of honey bee colonies were conducted on five apiaries (EA1–EA5) situated at five different geographical and climatic locations across Croatia. The aim of this study was to assess the comparative efficacy of CheckMite+ (Bayer, Germany), Apiguard (Vita Europe Ltd.; England), Bayvarol, C, (Bayer, Germany), Thymovar, (Andrma BioVet GmbH, Germany), and ApiLife Var, (Chemicals Laif SPA; Vigonza, Italy) for controlling the honey bee obligatory parasitic mite *Varroa destructor* in different conditions in the field during summer treatment. The relative varroa mite mortality after treatments with applied veterinary medicinal products were EA1 (59.24%), EA2 (47.31%), EA3 (36.75%), EA4 (48.33%), and EA5 (16.78%). Comparing the relative efficacy of applied varroacides, the best effect was achieved with CheckMite+, and the lowest for honey bee colonies treated with Apiguard (statistically significant difference was confirmed; $p < 0.05$). Considering the lower efficacy of thymol-based veterinary medicinal products observed on all EA in these study conditions, it may be concluded that their use is limited under different treatment regimes. Despite unfavourable weather and environmental conditions, with exceptions of EA5/EA5' and EA1, the relative varroacidal efficacy of authorized veterinary medicinal product treatments in moderately infested honey bee colonies ensured normal overwintering and colony development during next spring.

Keywords: *Apis mellifera*; *Varroa destructor*; experimental apiaries; varroacidal efficacy; VMP

1. Introduction

Honey bee colonies are valuable ecological and economical insects. They are fundamental for the food production and ecosystem biodiversity through their important role in pollination of cultivated crops and wild plants [1,2]. Different adverse environmental conditions, including pathogens and pests, have been implicated in the dissipated health of honey bee colonies or even their losses [3]. Although causes of extensive honey bee colony losses appear to be multifactorial [4], the obligate ectoparasitic mite *Varroa destructor* of honey bees is a major contributing factor [5]. As the *V. destructor* mite feeds on the haemolymph and fat body of adults [6] and developing stages of honey bees, and additionally facilitates the transmission of certain viruses [7–9], this seems to have a significant negative effect on the host immune response [10]. Because the aforementioned possible consequences of mite infestation consist in damages on the individual and colony level, the *V. destructor* mite population in honey bee colonies requires regular control during the whole year.

Moreover, honey bee colonies are highly influenced by beekeepers' management practices, socioeconomic conditions, and the level of implementation of policies supporting beekeeping

activities [11] in the particular country. In view of the spread of varroosis across Europe and the problems which this disease has brought in the beekeeping sector, the European Union (EU) has been encouraged to set up national programs aimed at improving the general conditions for the sustainable beekeeping production and management in its ecological, economic, and social dimensions [12].

In Croatia, as a new member of the EU, such apicultural programs have existed for several years. These national beekeeping programs (2011–2013, before accessing EU; 2014–2016; 2017–2019) employed a high participation rate of beekeepers [13] but failed to a certain extent in terms of the efficacy of the varroosis control by using authorized veterinary medicinal products (VMP). This is followed by the beekeepers' explanation that there are very few acaricide active substances on the market [14]. This is evident from the fact that in the past, beekeepers have not made any kind of notes or records of evidence regarding the use of varroacides as active ingredients and its efficacy on *V. destructor* mites. Over a few consecutive years, there has been a visible decreasing trend in procuration of authorized VMPs for usage in beekeeping [14], despite their total number, as well as the number of active substances, increasing by more than double [15].

According to the Varroa Control Program, which is a part of the Croatian National Regulation on animal protection measures against infectious and parasitic diseases and related financing from 2011 till today (with annual minor modifications) [16], beekeepers nationwide are required to implement one obligatory treatment of honey bee colonies against *V. destructor* mites using an authorized VMP in combination with other measures of integrated pest management implemented in an appropriate time of year [17]. Application of VMPs is recommended in the period from 1 July to 31 August, after main honey harvesting, and with the schedule dependent on the geographical, climatic and honey bee forage conditions in different regions. The main model is also to conduct treatments on all colonies and apiaries in the same area and during the same period. In this way, reinfestation and horizontal mite transfer between apiaries during robbing behavior [18,19] can be avoided.

Experimental apiaries (EA) in Croatia were established in 2013 with the purpose of conducting different research studies with a possible comparison of results linked to different environmental impacts.

There are numerous active acaricidal substances available incorporated into different formulations of medicines, different methods of application, and techniques for *V. destructor* mite population control. A variety of synthetic varroacides have been widely used in the last few decades with variable effects, due to geographical regions or often in response to bad beekeeping practices, such as multiple consecutive and repeated use of the same acaricide, sub- or overdosage, too short or prolonged treatment duration, improper time and way of acaricidal product application, too few active substances in the same time treatment, etc., which has led to increased tolerance to most of them. *V. destructor* mites have developed resistance to the most widely used synthetic varroacides [20–22]. In order to avoid the accumulation of chemical residues in honey and beeswax, beekeepers are very interested in treatments of natural, ecofriendly, so-called *soft* acaricides, which are often inconsistent, more variable, or effectiveness or therapeutically limited under different climatic conditions [23–26].

The aim of this study was to evaluate and compare the varroacidal efficacy and mite mortality dynamic during summer treatment of honey bee colonies situated at EA influenced by different environmental conditions, treated with five different available authorized VMPs (CheckMite+, Apiquard, Bayvarol, Thymovar, and ApiLife Var) used simultaneously and compared with negative control (untreated group of honey bee colonies). The percentages of *V. destructor* mite mortality by the experimental treatments were estimated according to recommendations of the European Medicines Agency (EMA) [27]. Efficacy of treatments was also compared between EA. Additionally, the commonly used oxalic acid was used for follow-up winter treatment in broodless honey bee colonies to establish the final parasitic mite drop.

2. Materials and Methods

2.1. Locations of Experimental Apiaries and Field Trail Design

The study was conducted during active beekeeping season of 2014 at five different apiaries: EA1–EA5 (Figure 1; http://geoportal.dgu.hr/) in Croatia. EAs were located in different geographical regions: EA1 (46° 5′ 37″ N, 15° 53′ 34″ E); EA2 (45° 48′ 16″ N, 18° 39′ 54″ E); EA3 (45° 13′ 45″ N, 13° 56′ 29″ E); EA4 (44° 36′ 54″ N, 43° 55′ 46″ E), and EA5 (43° 55′ 47″ N, 16° 26′ 18″ E). EA1 was situated in grassland surrounded by fields where intensive agriculture practice is in use; EA2 was on the lea surrounded with vegatable gardens; EA3 was located in a rural area surrounded with orchards and vineyards; EA4 was in the deciduous wood, and EA5 was situated on the grassland prairie. Due to the lack of natural food during July and August on the EA5 location, the honeybee colonies were moved after summer treatment against *V. destructor* mites to a new, more favourable location on the Adriatic sea island Vis. The new position of this apiary was annotated as EA5′ (43° 2′ 45″ N, 16° 9′ 14″ E).

Figure 1. Locations of experimental apiaries in Croatia.

Each EA consisted of 30 honey bee colonies (*Apis mellifera carnica* Pollmann, 1879) placed in standard Langsthrot Root (LR) hives. Experimental colonies were queen right, had combs occupied with adult honey bees, and were fully developed and productive. Prior to the experimental period, all experimental honey bee colonies were uniformed in respect to brood size, the comb area covered with adult bees, and amount of stored food [28]. Honey bee queens were one year old. Experimental colonies were also visually inspected for the presence of pathology signs on adult bees and brood. Adult bees showed normal behavior, and there were no visible signs of infectious diseases on brood. No acaricidial treatment of the honey bee colonies was done prior to the start of experimental treatments. After inspection, the honeybee colonies situated on EA1–EA5 were divided into six experimental groups (A, B, C, D, E, and O), and each group consisted of five beehives (Table 1).

All beehives were equipped with varroa mite screen boards for monitoring mite fall counts. In early spring, metal sheets were placed on the bottom board of each bee hive in order to record the natural mite mortality prior to treatments, and later in the season, the mite drop after the experimental treatments. Above the sheets, wire screens were installed to prevent contact of the adult bees with debris and to prevent ants from removing dropped *V. destructor* mites. Experimental and control colonies were monitored for mites mortality in prior, during, and after treatments performed in brood and broodless (winter) periods. Mite counts were carried out every day during summer treatment (A, C, D, and O groups—42 reads; B and E groups—28 reads were recorded for each colony), and seven days after winter treatment. At EA5′, the emergency autumn treatment was performed on 16 September by the commonly used amitraz on a one-time basis.

Table 1. Field trail design.

Apiary	Pretreatment Mite Fall *n* = 150	CheckMite+ A *n* = 5/apiary	Apiguard B *n* = 5/apiary	Bayvarol C *n* = 5/apiary	Thymovar D *n* = 5/apiary	ApiLife Var E *n* = 5/apiary	Oxalic Acid A,B,C,D,E,O
EA1	1.6–24.7	25.7–5.9	25.7–22.8	25.7–5.9	25.7–5.9	25.7–22.8	28.11–6.12
EA2	1.6–17.7	17.7–28.8	17.7–14.8	17.7–28.8	17.7–28.8	17.7–14.8	29.11–7.12
EA3	1.6–15.7	16.7–27.8	16.7–13.8	16.7–27.8	16.7–27.8	16.7–13.8	11.12–18.12
EA4	1.6.–16.7	17.7–28.8	17.7–14.8	17.7–28.8	17.7–28.8	17.7–14.8	1.12–8.12
EA5	1.6–14.7	15.7–26.8	15.7–12.8	15.7–26.8	15.7–26.8	15.7–12.8	_

Note: On every experimental apiary (EA), the negative control was included (untreated group of honeybee colonies, O; *n* = 5/apiary); treatment of broodless honeybee colonies with oxalic acid was carried out on all survivals during the winter period, except in EA5.

2.2. Drugs and Treatments

Treatments were conducted during the summer season immediately after the main honey harvesting. The authorized VMPs were used in the recommended doses. CheckMite+ (Bayer, Leverkusen, Germany), based on coumaphos as the active ingredient, was applied in the form of two beehive pest control strips inserted between frames, with waxcombs sealed with honeybee brood in the brood chamber for a 42 day period. Apiguard (Vita Europe Ltd.; Basingstoke, England) is an authorized thymol-based acaricide packed in an aluminum tray, so its coated sheets were placed on the top bars of the bee hive frames of each brood chamber, one tray during two weeks and the second one during the consecutive two weeks. The treatment with flumethrin in an authorized VMP—Bayvarol (Bayer)—was applied as four pest control strips were inserted between frames with sealed honey bee brood in the brood hive chamber for a 42 day period. Thymovar (Andermatt BioVet GmbH, Lörrach, Germany), formulated on cellulose wafer, contains 15 g of thymol and was used as one piece cut in two parts, which were separately placed on the top bars of frames on a two-time-basis for 42 days in total. ApiLife Var (Chemicals Laif SPA; Vigonza, Italy) is based on a few active ingredients (thymol, eucalyptus oil, levomenthol, and camphor) imbibed in vermiculite tablets. One tablet per bee hive was applied every eight days with four applications in total. Every portion of the tablet was placed in a corner of the brood hive chamber and remained in the honey bee colonies to be chewed and removed by the adult bees.

The oxalic acid solution was prepared using 1 L of sugar syrup to dissolve 35 g of oxalic acid dehydrate (Kemika, Zagreb, Croatia). The sugar syrup was prepared by mixing hot freshwater (70–80 °C) with commercial sugar (Viro, Virovitica, Croatia) (1:1). Prepared oxalic acid solution was administered to the honey bee colonies cold with a syringe trickling 5 mL for each intercomb space occupied by adult bees, from the top. The number of phoretic mites fallen after the winter treatment was counted by using metal label sheets on the bottom boards, checked every day for one week.

For autumn treatment, Varidol (TolnAgro Kft., Szekszárd, Hungary) was used once, in honeybee colonies at location EA5', by fumigation according to instructions for use.

2.3. Meteorological Conditions

Data on weather conditions (air temperature (°C), relative air moisture (%), number of days with rain; average values per month) during monitored beekeeping season were obtained from Croatian Hydrometeorological Department, from local climatic-meteorological stations (Osijek, Krapina, Pazin, Gospić, Sinj). All parameters were measured three times per day at 7:00 a.m., 2:00 p.m., and 9:00 p.m.

2.4. Estimating the Strength of Honeybee Colonies

To estimate the strength of honey bee colonies, a Liebefeld method was performed, with visual determination of number of adult honey bees and brood sealed in beeswax combs [28]. The estimation

of honey bee colonies was conducted three times (prior—I, during—II, and after experimental summer treatments—III), during morning hours, between 9:00 and 10:00 a.m. before the first massive forage flights of honey bees. Strength of honey bee colonies was estimated as follows: EA1—19 May, 25 July, and 16 September; EA2—29 April, 17 July, and 8 September; EA3—14 May, 16 July, and 7 September; EA4—20 May, 10 July, and 2 September; and EA5—28 April, 15 July, and 10 September. Owing to easier assessment with adult honey bees or brood-covered comb areas, the frame for the LR hive was used and prior divided with a plastic grid into 1 dm^2 quadrants.

2.5. Colony Examinations, Mite Counts and Treatments Efficacy

Clinical examination of honeybee colonies included visual inspection of honeybee brood and adult bees, the behaviour of adult bees, as well as activity of bees at the entrances of hives. Fallen *V. destructor* mites were counted during the pretreatment, treatment, and a particular number of days after each treatment, and the sum of those results calculated after the final treatment was considered the total mite drop. The proportion of mites falling after each treatment to the total number of fallen mites was estimated in percentages (%). The efficiency of each experimental treatment (A, B, C, D, E) was estimated according to the recommendations of the EMA [27].

2.6. Data Analysis

The data analyses were performed by one-way analysis of variance (ANOVA) using statistical software package Statistica-StatSoft v.7. (StatSoft, Inc, Tulsa, OK, U.S.A.). The results were presented as the mean values, standard deviations, and standard errors. To assess the statistical differences in honeybee colonies' strength (estimated number of honeybees per colony), they were compared between groups and three estimation dates (April/May, July, and September), and the number of fallen *V. destructor* mites between experimental groups (A, B, C, D, E) and control group (O) at different locations and estimation dates, the Kruskal–Wallis test was performed ($\alpha = 0.05$). Multisample comparison using Kruskal–Wallis H test was carried out to compare the mean values of the number of fallen *V. destructor* mites between experimental and control groups in the pretreatment, treatment, and after-treatment period.

3. Results

3.1. Meteorological Conditions

The average values of air temperatures, relative air moisture, and number of days with rain for each location of EA during the observed period of beekeeping season 2014 is shown in Table 2. Obviously, these harms encountered in beekeeping may indicate that this acaricidal therapy of honey bee colonies with authorised VMPs had the obtained effect instead of especially detrimental environmental circumstances.

Table 2. Meteorological conditions at different apiaries.

Meteorological Circumstances (Average Values Per Month)															
Month	**Air Temperature (°C)**					**Relative Air Moisture (%)**					**Number of Days with Rain**				
	EA1	**EA2**	**EA3**	**EA4**	**EA5**	**EA1**	**EA2**	**EA3**	**EA4**	**EA5**	**EA1**	**EA2**	**EA3**	**EA4**	**EA5**
Mar	9.5	9.9	8.9	7.0	9.5	74	65	67	69	68	9	8	5	10	9
Apr	13.2	12.9	11.9	10.5	12.6	74	73	74	72	71	11	12	8	10	15
May	16.1	14.9	14.4	12.9	15.1	73	69	72	66	69	11	18	3	7	14
Jun	20.5	19.2	19.6	17.7	20.1	67	74	66	67	66	3	9	2	4	10
Jul	21.8	20.7	20.0	18.6	21.1	74	76	78	73	73	3	10	6	10	12
Aug	20.8	19.1	19.4	18.3	20.9	76	79	79	72	73	6	11	8	2	7
Sep	17.0	15.6	15.7	13.7	16.7	82	86	81	84	79	10	17	11	13	14
Oct	13.3	12.8	13.0	11.2	13.7	82	85	83	79	76	9	9	8	7	10
Nov	8.3	8.6	11.0	8.5	10.9	87	89	87	86	84	3	17	16	13	14

3.2. Estimating the Strength of Honeybee Colonies

Despite the equalization of honeybee colonies before pretreatment period with respect to colony strength, some statistical differences were determined between experimantal and control groups of honey bee colonies at different estimation days (I, II, or III) at EA1, EA2, and EA5, as follows: EA1: I ($p < 0.001$; F = 9.87), II ($p < 0.05$; F = 3.32), III ($p < 0.01$; F = 3.94); E2: III ($p < 0.001$; F = 9.87); and E5: III ($p < 0.05$; F = 3.55). Variations in the average number of honey bees per group during three estimation terms are shown in Figure 2.

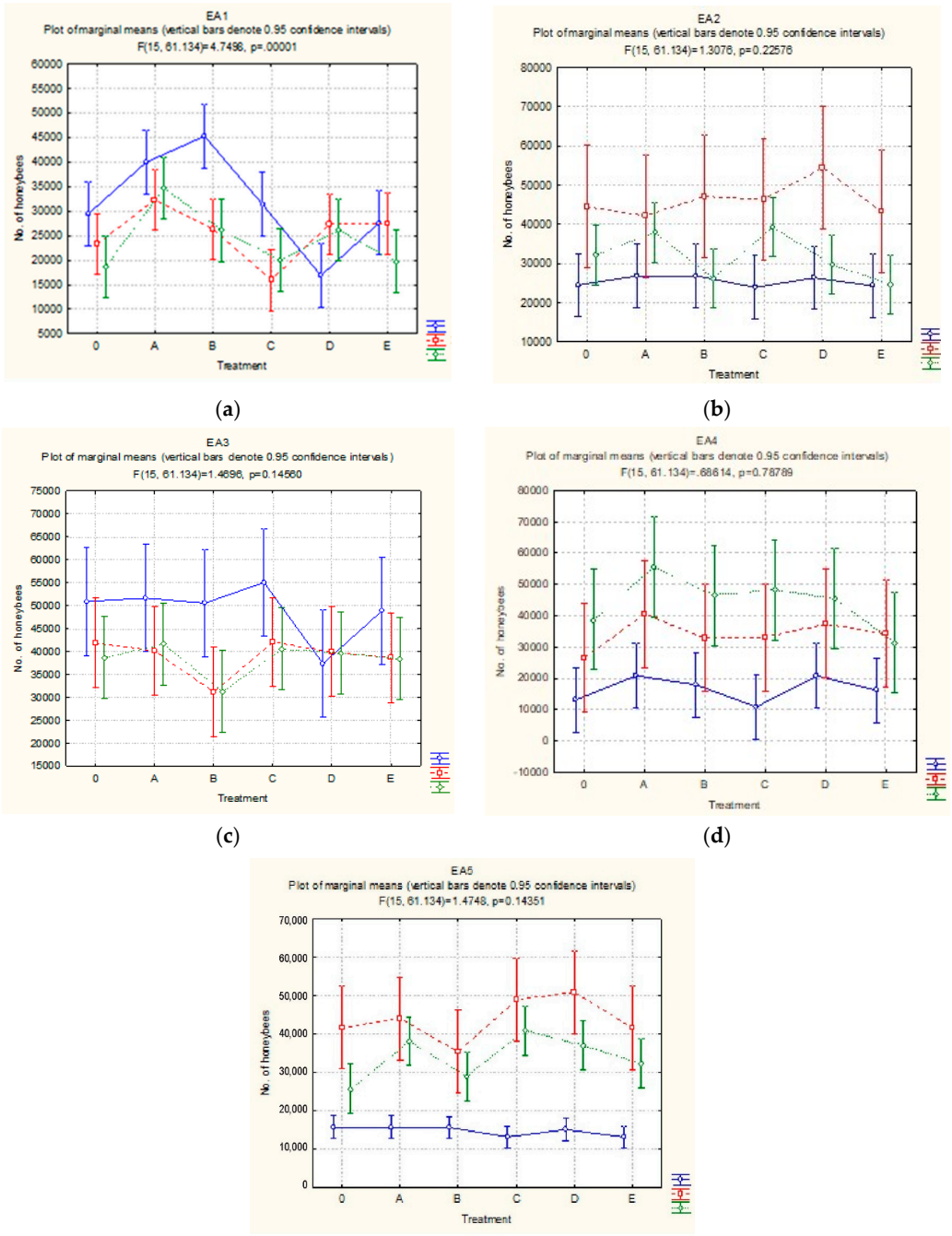


Figure 2. Honey bee colony strength differences between experimental and control groups by different estimation dates (I—blue lines, II—red lines, III—green lines) at five apiary locations: (**a**) EA1, (**b**) EA2, (**c**) EA3, (**d**) EA4, (**e**) EA5.

3.3. V. destructor Mite Fall Prior to, during, and after Varroacidal Treatments

3.3.1. Pretreatment Period

During the pretreatment periods the average daily mite drops in 30 honey bee colonies per apiary were: EA1, 2.76 (± 2.60); EA2, 3.04 (± 2.60); EA3, 0.80 (± 0.10); EA4, 4.32 (± 0.50); and EA5, 0.09 (± 0.10). These values did not differ significantly between the experimental groups on individual apiaries but were significantly different between EA locations (EA4, EA5; $p < 0.001$). Results are presented in Figure 3.

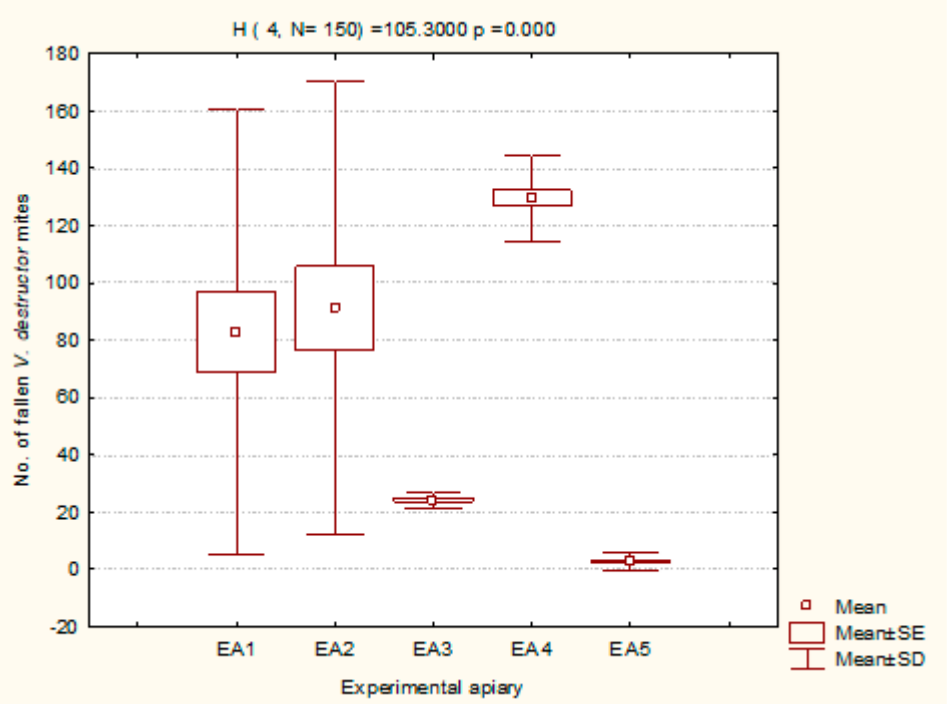


Figure 3. Natural *V. destructor* mite drop during the pretreatment period at five apiary locations (EA1–EA5).

3.3.2. Treatment Period

During the summer treatment period, the estimated total *V. destructor* mite mortality that resulted was significantly higher than natural mite drop. Results of summer and winter treatment (to determine the residual amount of mites) of honey bee colonies with VMPs are shown in Figure 4. Because of very low efficacy of summer treatments in all groups of experimental colonies situated on EA5 (Figure 5), the necessary follow-up autumn treatment was done at EA5′ (Figure 5), and calculations of treatments efficacy was based on those mite drops (A—47.52%; B—3.11%; C—25%; D —5.18%; E—3.11%). Results of this EA were analyzed separately from those of other EAs.

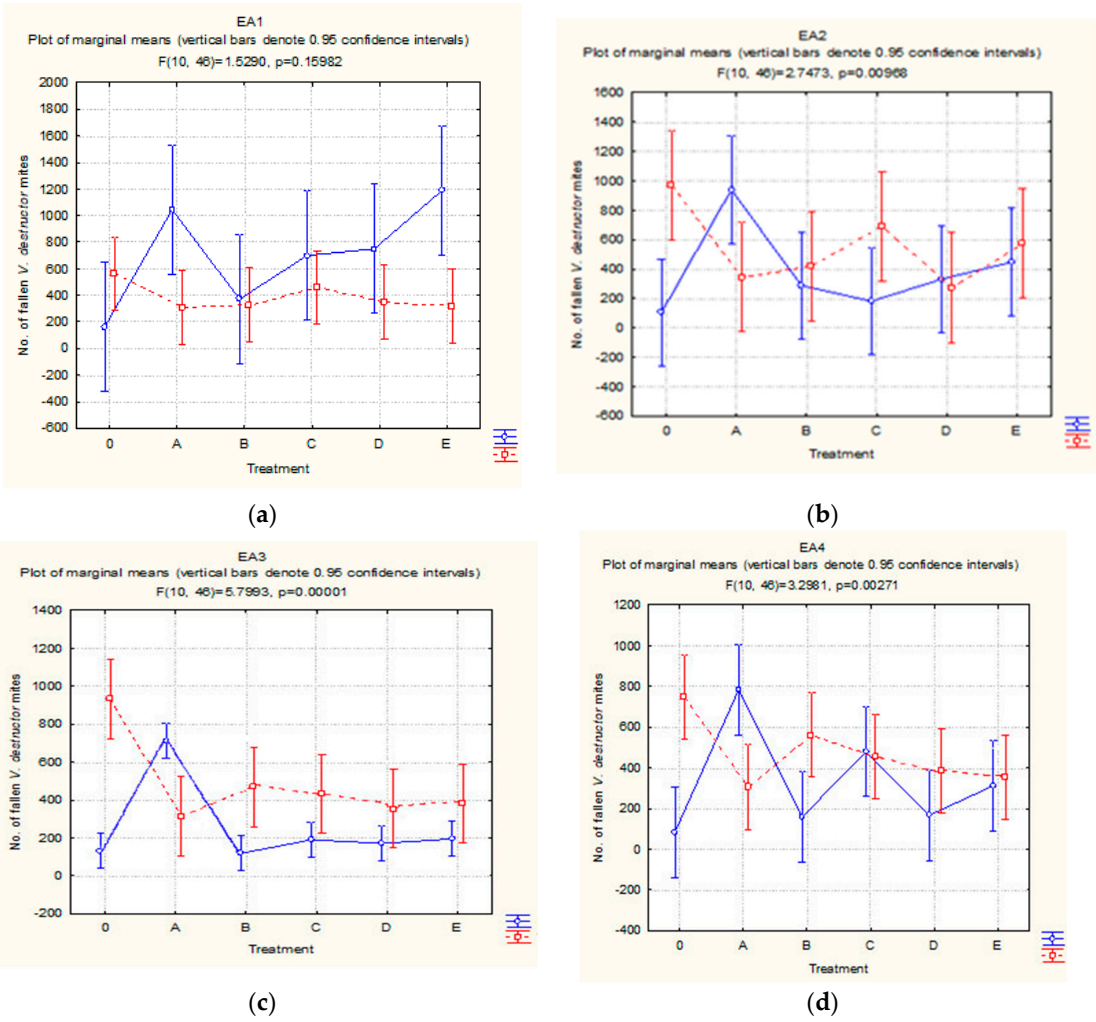


Figure 4. Differences in the number of fallen *Varroa destructor* mites between experimental groups (A, B, C, D, E) and control groups (O), during summer (blue lines) and winter treatment (red lines); mean ± SD. (**a**) EA1, (**b**) EA2, (**c**) EA3, (**d**) EA4.

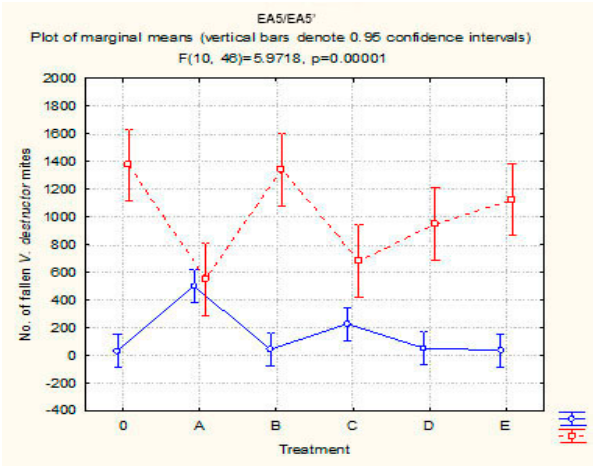


Figure 5. Differences in the number of fallen *V. destructor* mites between experimental groups (A, B, C, D, E) and control groups (O), during summer (blue lines) and follow-up autumn treatment (red lines), at EA5/EA5′; mean ± SD.

The mean values of *V. destructor* mortality on EA1 did not differ significantly between the experimental groups (A, B, C, D, E), but it was different in comparison with the control group ($p < 0.05$; h = 14.2). Summer treatment results were significantly different from those of winter treatment ($p < 0.05$; F = 2.74). Efficacy of varoacidal treatment is presented in Table 3, and it was decreased as follows: A > E > D > C > B. Prior to winter treatment, nine of the treated honey bee colonies were dead: 3 colonies from B group, 3 colonies from E group, 2 colonies from D group and 1 colony from C group.

Table 3. Treatment efficacy of varroacides on different experimental apiaries.

EA	Experimental Group	Number of Fallen *V. destructor* Mites (S/W)			% of Treatment Efficacy
-	-	Mean	Min.	Max.	
EA1	A	1044/286.4	430/237	1967/449	78.50
	B ‡‡‡	371.6/660.5	149/616	975/705	36.00
	C ‡	699.2/546	168/490	1112/609	56.20
	D‡‡	780/509.33	283/357	1605/774	60.50
	E ‡‡‡	1188.8/642	429/604	2046/680	65.00
EA2	A	950/326.6	381/135	2217/671	74.42
	B	287.8/421.2	65/177	744/658	40.60
	C	200.4/680.8	96/111	326/1038	22.75
	D	332.2/273.6	152/85	664/638	54.90
	E	448.4/572.8	48/100	1177/1195	43.90
EA3	A	724/314	603/138	802/435	69.75
	B	118.4/489.2	97/233	159/635	19.49
	C	190.4/433.4	115/231	346/625	30.52
	D	171.6/379.6	114/168	333/589	31.13
	E	194.6/397	102/168	442/602	32.90
EA4	A	783.2/264.4	125/278	1207/382	74.77
	B	195/542.4	43/384	381/789	26.45
	C	480.2/405.2	279/240	766/563	54.24
	D	167.2/406.6	48/180	373/594	41.13
	E ‡	312.6/380.7	30/126	1119/594	45.09

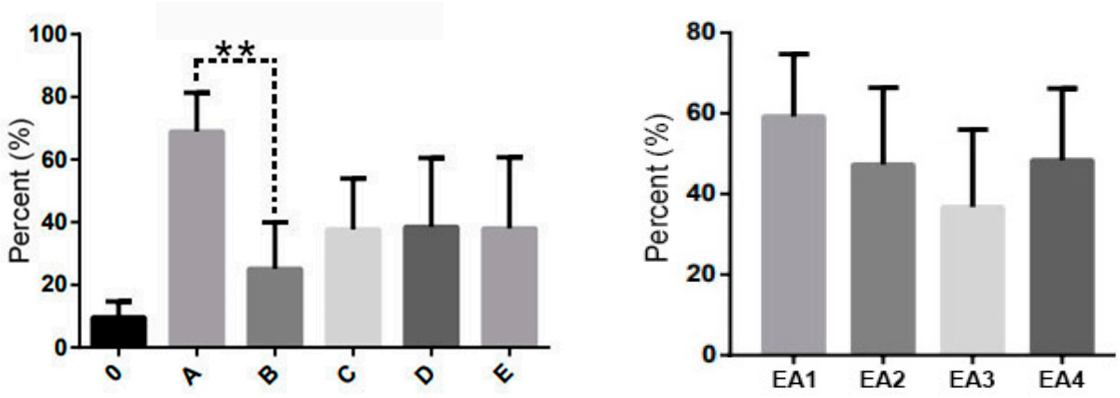
Note: S—summer treatment; W—winter treatment; ‡, ‡‡, ‡‡‡—number of dead honey bee colonies prior to winter treatment, in particular experimental groups.

At the location of EA2, each treatment (A, B, C, D, E) induced a significantly higher ($p < 0.05$; h = 12.9) *V. destructor* mite mortality in the parallel untreated control colonies (O). The summer treatments and winter treatments ($p < 0.005$; F = 2.83) were also statistically significantly different. Efficacy of different VMP treatments (Table 3) decreased as follows: A > D > E > B > C. All honey bee colonies survived.

The mortality rates in the treated colonies of EA3 apiary were significantly different from those in the control honey bee colonies ($p < 0.05$; h = 12.77), and between the summer and winter treatments ($p < 0.001$; F = 5.79). Efficacy of acaricidal treatments (Table 3) decreased in the same order as on the EA1.

Total V. destructor mite drop after the treatments were significantly higher ($p < 0.005$; h = 16.6) than number of dead mites counted on the bottom boards hive inserts of untreated control honey bee colonies situated at EA4. Also, the differences between experimental groups were determinated ($p < 0.01$; F = 5.95), during the summer treatments. Order of VMPs treatment efficacy decreased as follows: A, C, E, D, and B.

Varroacidal efficacy of applied VMPs was significantly different for treatments of experimental honey bee colonies from groups A and B (Figure 6a), at each EA ($p < 0.001$, F = 6.933). As a consequence of nontreated honey bee colonies during the summer period, all control groups were also significatly lower regarding percentages of varroacidal efficacy. Overall varroacidal efficacy at individual EA did not differ significantly between EA1, EA2, EA3, and EA4. These results are presented in Figure 6b.



(**a**) (**b**)

Figure 6. Treatment efficacy of varroacides with different active ingredients; (**a**) treatments; (**b**) locations; ** ($p < 0.05$); mean ± SD.

4. Discussion

Varroa mites, acting simultaneously with other stressors, are known to be a main cause of honey bee colony weakening and collapsing [5,29]. The *V. destructor* mite population requires regular control, correctly and in a timely manner, of honey bee colony management. Five different VMPs, only authorized in Croatia in 2014, were tested at five apiaries to test their performance in different field conditions because control of varroosis by using VMPs is widely acknowledged to be an essential part of beehive management [30].

The 2014 active beekeeping season was generally very weak in different aspects. There were more days with rain, very low average year air temperatures, along with a mild winter, and consequently, a very early honey bee brood development was noticed, even in January. As one replication cycle of *V. destructor* mites is about 13 days, multiple replications of mites can be expected to have occurred [17]. Weather circumstances were also very favorable for a fast-increasing growth trend of *V. destructor* mite populations in honey bee colonies with early reared brood [31]. Additionally, lack of natural food in the environment effected a decreasing of immunological status of the honey bee colonies and induced stress caused by hunger. All these detrimental impacts of environmental factors, high *V. destructor* mite infestations, and weak main pastures ended very early, so in most regions, the first summer varroacidal treatment was essential.

A different number of *V. destructor* mite drops was detected during the pretreatment period, which was probably a consequence of a different level of parasitic colony invasions at different EAs. Meeting the demand of no varroacidal treatments that year, before the start of the described study means that they were treated the last time in the winter preceding the experimental period. This may have implicated a situation where establishing new summer generations in all honey bee colonies coincided with a high level of *V. destructor* mite infestation, which may have damaged colonies in each experimental group. Similar findings were published before [32].

Because of extremely low amounts of stored honey in hives, at EA2 and EA5, the honey extraction was not done for the whole year.

Treatments against varroa mites started at different dates for each EA because the blooming of plants that make up the main bee pastures finished at different times. The best relative varroacidal efficacy was seemingly achieved at EA1 (59.24%), but if an analysis of the survival of treated colonies is included, then it is clear that 30% of them collapsed before the next active beekeeping season. Most of the lost honey bee colonies were treated with VMPs based on thymol as active ingredients. Here it must be stressed that first summer treatments on EA1 started only towards the end of July, which is relatively late but still in accordance with advised varroosis control schedules in Croatia.

Although relative average treatment efficacy at EA2 (47.31%), EA3 (36.75%), and EA4 (48.33%) was pretty low and below expectations, all honey bee colonies survived and successfully overwintered (except one honey bee colony at EA4, treated with a thymol-based VMP).

Comparing the relative efficacy of the used varroacides with different active ingredients (Figure 6a), the best effect was achieved for honey bee colonies from an A experimental group, and lowest for colonies from a B experimental group, where statistically significant difference was affirmed ($p < 0.05$; $F = 6.93$). Although the organic acaricides have certain advantages after repeated use, their efficacy may be inconsistent and more variable compared with synthetic acaricide formulations [24,33–36], which is also confirmed with our results.

Changes in honey bee colonies' strength trends were different between apiary locations, but within the expected ranges under the study and environmental conditions, as well as beekeeping practices (EA1, EA2, EA3, and EA4).

At location EA5 at the beginning of active beekeeping season, honey bee colonies were very weak with low numbers of adult bees (Figure 2). Then, in the middle of the active season, there was an opposite situation: a high number of adult bees without brood or very few comb cells were sealed with development stages of bees. At the same time there was non food in nature, and honey bees ate away almost all food storages in their hives. After emergency varroacidal treatment, which produced higher efficacy compared to earlier treatments because larger proportion of phoretic mites being on adult bees [37], and transport at island Vis (EA5′), they came in with much better environmental circumstances with plenty of natural food. In the next few weeks, the strength of colonies increased quickly, but because of serious damages, bees got high varroa infestation and insufficient efficacy of VMPs treatment during the summer treatment (except A group—47.52%), most of the colonies from other treated groups died before the next spring (63.4%). The efficacy of the VMP treatments was under expectations, probably due to fast reproduction of the surviving mites, but also because of possible reinfestations during experiments [35].

5. Conclusions

Varroa mite infestations control using CheckMite+ (Bayer, Germany), Apiguard (Vita Europe Ltd.; England), Bayvarol (Bayer, Germany), Thymovar (Andrma BioVet GmbH, Germany), and ApiLife Var (Chemicals Laif SPA; Vigonza, Italy) at five different EAs in Croatia during the beekeeping season of 2014 induced a higher mite mortality compared to control, as well as in comparison with mite drop in the pretreatment period. Despite unfavourable weather and environmental conditions, with the exceptions of EA5/EA5′ and EA1, the relative varroacidal efficacy of authorized VMP treatments in moderately infested colonies ensured normal overwintering and colony development during the next spring. Due to a lower efficacy of thymol-based VMPs observed at all EAs in this study conditions, it may be concluded that their use is limited under different treatment regimes. The results of this study imply that efficacy of used varroacidals strongly depends on geography, but also on timely manner, and can vary from season to season.

It can be concluded that an adequate *V. destructor* mite control must include a few measures, primarily good beekeeping maintenance techniques in combination with appropriate use of authorized VMPs. Different treatment regimes should also be applied with continuous parasitic mite mortality monitoring. Application of varroacides should be performed after the main honey flow, on all apiaries of the same epizootiology area, and in all honey bee colonies with mite infestations levels above the

economic threshold. The same and appropriate treatment timing will ensure honey bee colonies surviving and prevent reinfestations. In specific situations, it is possible to use emergency treatments and alternate synthetic acaricides with food additives with acaricidal effect in rotation programs in order to decelerate the resistance of varroa mites to multiply used acaricides and to reduce the impact of increasing comb wax contamination. All varroacidal treatments must be performed in accordance and in combination with other specific regulations ordered by the national authorities.

Author Contributions: Conceptualization, I.T.G.; methodology, I.T.G.; formal analysis I.T.G. and L.S.; investigation, I.T.G., L.S., D.B., and T.Ž.; resources, I.T.G. and D.B.; data curation, L.S. and I.T.G.; writing—original draft preparation, I.T.G.; writing—review and editing, L.S., D.B., T.Ž., and I.T.G.; visualization, L.S. and I.T.G. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Project—Impact of diseases epidemiology and environmental factors to the biological-rearing status of honey bee colonies; Croatian National Beekeeping Program 2014.

Acknowledgments: The authors would like to thank colleagues Saša Prđun and Josipa Vlainić for technical assistance; and beekeepers Dario Dješka, Ranko Anđelini, Ivan Balenović, and Antionio Mravak for technical support and maintenance of the experimental apiaries.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

References

1. Poots, S.G.; Biesmeijer, J.C.; Kremen, C.; Neumann, P.; Schweiger, O.; Kunin, W.E. Global pollinator declines: Trends, impacts and drivers. *Trends Ecol. Evol.* **2010**, *25*, 345–353. [CrossRef] [PubMed]
2. Rollin, O.; Benelli, G.; Benvenuti, S.; Decourtye, A.; Wratten, S.D.; Canale, A.; Desneux, N. Weed-insect pollinator networks as bio-indicators of ecological sustainability in agriculture. A review. *Agron. Sustain. Dev.* **2016**, *36*, 1–22. [CrossRef]
3. Goulson, D.; Nicholls, E.; Botias, C.; Rotheray, E.L. Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science* **2015**, *347*, 1255957. [CrossRef] [PubMed]
4. Kluser, S.; Neumann, P.; Chauzat, M.P.; Pettis, J.S. UNEP Emerging Issues: Global Honey Bee Colony Disorder and other Threats to Insect Pollinators. United Nations Environment Programme 2010. Available online: https://europa.eu/capacity4dev/unep/document/global-bee-colony-disorders-and-other-threats-insect-pollinators (accessed on 30 October 2019).
5. Smith, K.M.; Loh, E.H.; Rostal, M.K.; Zambrana-Torrelio, C.M.; Mndiola, L.; Daszak, P. Pathogens, pests, and economics: Drivers of honey bee colony declines and losses. *EcoHealth* **2014**, *10*, 434–445. [CrossRef] [PubMed]
6. Ramsey, S.D.; Ochoa, R.; Bauchan, G.; Gulbronson, C.; Mowery, J.; Cohen, A.; Lim, D.; Joklik, J.; Cicero, J.M.; Ellis, J.D.; et al. *Varroa destructor* feeds primarily on honey bee fat body tissue not haemolymph. *Proc. Natl. Acad. Sci. USA* **2019**, *116*, 1792–1801. [CrossRef]
7. Ryabov, E.V.; Wood, G.R.; Fannon, J.M.; Moore, J.D.; Bull, J.C.; Chandler, D.; Mead, A.; Burroughs, N.; Evans, D.J. A Virulent Strain of Deformed Wing Virus (DWV) of Honeybees (*Apis mellifera*) Prevails after *Varroa destructor*-Mediated, or *in Vitro*, Transmission. *PLoS Pathog.* **2014**, *10*, e1004230. [CrossRef] [PubMed]
8. Nazzi, F.; Brown, S.P.; Annoscia, D.; Del Piccolo, F.; Di Prisco, G.; Varricchio, P.; Della Vedova, G.; Cattonaro, F.; Caprio, E.; Pennacchio, F. Synergistic parasite-pathogen interactions mediated by host immunity can drive the collapse of honey bee colonies. *PLoS Pathog.* **2012**, *8*, e1002735. [CrossRef]
9. Nazzi, F.; Le Conte, Y. Ecology of *Varroa destructor*, the Major Ectoparasite of the Western Honey Bee, *Apis mellifera*. *Annu. Rev. Entomol.* **2016**, *61*, 417–432. [CrossRef]
10. Yang, X.; Cox-Foster, D.L. Impact of an ectoparasite on the immunity and pathology of an invertebrate: Evidence for host immunosuppression and viral amplification. *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 7470–7475. [CrossRef]
11. Gilioli, G.; Simonetto, A.; Hatina, F.; Sperandio, G. Multi-dimensional modelling tools supporting decision-making for the beekeeping sector. *IFAC PapersOnLine* **2018**, *51*, 144–149. [CrossRef]

12. Rortais, A.; Arnold, G.; Dorne, J.-L.; More, S.J.; Sperandio, G.; Streissl, F.; Szentes, C.; Verdonck, F. Risk assessment of pesticides and other stressors in bees: Principles, data gaps and perspectives from the European Food Safety Authority. *Sci. Total Environ.* **2017**, *587*, 524–537. [CrossRef] [PubMed]
13. Anonymous. *National Beekeeping Program for Period 2017–2019*; Ministry of Agriculture: Zagreb, Croatia, 2016.
14. Anonymous. *National Beekeeping Program for Period 2020–2022*; Ministry of Agriculture: Zagreb, Croatia, 2019.
15. Anonymous. *Program for Varroosis Control and Eradication*; Ministry of Agriculture: Croatia, 2017. Available online: http://www.veterinarstvo.hr/default.aspx?id=140 (accessed on 2 November 2019).
16. Anonymous. *Croatian National Regulation on Animal Protection Measures against Infectious and Parasitic Diseases and on Related Financing for 2019*; Ministry of Agriculture: Croatia, 2019. N.N. 5/2019. Available online: https://narodne-novine.nn.hr/clanci/sluzbeni/2019_01_5_92.html (accessed on 2 November 2019).
17. Rosenkranz, P.; Aumeier, P.; Ziegelmann, B. Biology and control of *Varroa destructor*. *J. Invertebr. Pathol.* **2010**, *103*, S96–S119. [CrossRef] [PubMed]
18. Frey, E.; Rosenkranz, P. Autumn invasion rates of *Varroa destructor* (Mesostigmata: Varroidae) into honey bee (Hymenoptera: Apidae) colonies and the resulting increase in mite populations. *J. Econ. Entomol.* **2014**, *107*, 508–515. [CrossRef] [PubMed]
19. Seeley, T.D.; Smith, M.L. Crowding honeybee colonies in apiaries can increase their vulnerability zo the deadly ectoparasite *Varroa destructor*. *Apidologie* **2015**, *46*, 716–727. [CrossRef]
20. Floris, I.; Satta, A.; Garau, V.L.; Melis, M.; Cabras, P.; Aloul, N. Effectiveness, persistence, and residue of amitraz plastic strips in the apiary control of *Varroa destructor*. *Apidologie* **2001**, *32*, 577–585. [CrossRef]
21. Sammataro, D.; Untalan, P.; Guerrero, F.; Finley, J. The resistance of varroa mites (Acari: Varroidae) to acaricides and the presence of esterase. *Int. J. Acarol.* **2005**, *31*, 67–74. [CrossRef]
22. Maggi, M.D.; Ruffinengo, S.R.; Negri, P.; Eguaras, M.J. Resistance phenomena to amitraz from populations of the ectoparasitic mite *Varroa destructor* of Argentina. *J. Parasitol. Res.* **2010**, *107*, 1189–1192. [CrossRef]
23. Howis, M.; Nowakowski, P. *Varroa destructor* removal efficiency using Beevital Clean preparation. *J. Apic. Sci.* **2009**, *53*, 15–20.
24. Rodriguez-Dehaibes, S.R.; Parido Sedas, V.T.; Luna-Olivars, G.; Villanuva-Jimenez, J.A. Two commercial formulations of natural compounds for *Varroa destructor* (Acari: Varroidae) control on Africanized bees under tropical climatic conditions. *J. Apic. Res.* **2017**, *56*, 58–62. [CrossRef]
25. Tlak Gajger, I.; Sušec, P. Efficacy of varroacidal food additive appliance during summer treatment of honeybee colonies (*Apis mellifera*). *Vet. Arhiv* **2019**, *89*, 87–96. [CrossRef]
26. Tlak Gajger, I.; Tomljanović, Z.; Stanisavljević, L. An environmentally friendly approach to the control of *Varooa destructor* mite and *Nosema ceranae* disease in Carnolian honeybee (*Apis mellifera carnica*) colonies. *Arch. Biol. Sci.* **2013**, *65*, 1585–1592. [CrossRef]
27. Anonymous. Guideline on Veterinary Medicinal Products Controlling *Varroa Destructor* Parasitosis in Bees. EMA— European Medicines Agency, EMA/CVMP/EWP/459883/2008, Committee for Medicinal Products for Veterinary Use (CVMP). Available online: http://www.ema.europaeu/docs/en_GB/document_library/Scientific_guideline/2010/11/WC500099137.pdf (accessed on 2 November 2019).
28. Delaplane, K.S.; Van Der Steen, J.; Guzman-Novoa, E. Standard methods for estimating strength parameters of *Apis mellifera* colonies. *J. Apic. Res.* **2013**, *52*, 1–12. [CrossRef]
29. Tlak Gajger, I.; Tomljanovic, Z.; Petrinec, Z. Monitoring health status of Croatian honey bee colonies and possible reasons for winter losses. *J. Apic. Res.* **2010**, *49*, 107–108. [CrossRef]
30. Mutinelli, F. Veterinary medicinal products to control *Varroa destructor* in honey bee colonies (*Apis mellifera*) and related EU legislation—An update. *J. Apic. Res.* **2016**, *55*, 78–88. [CrossRef]
31. Žorat, T. *The Efficiency of Acaricides used in Summer Treatment of Honeybee Colonies against Varroosis. Graduating Thesis*; University of Zagreb Faculty of Veterinary Medicine: Zagreb, Croatia, 10 October 2015.
32. Van Dooremalen, C.; Gerritsen, L.; Cornelissen, B.; van der Steen, J.J.M.; van Langevelde, F.; Blacquière, T. Winter Survival of Individual Honey Bees and Honey Bee Colonies Depends on Level of *Varroa destructor* Infestation. *PLoS ONE* **2012**, *7*, e36285. [CrossRef] [PubMed]
33. Gregorc, A.; Smodis Škerl, I.M. Combating *Varroa destructor* in Honeybee Colonies Using Flumethrin or Fluvalinate. *Acta Vet. Brno* **2007**, *76*, 309–314. [CrossRef]
34. Gregorc, A.; Planinc, I. Use of Thymol formulations, Amitraz, and Oxalic Acid for the control of the varroa mite in honey bee (*Apis mellifera carnica*) colonies. *J. Apic. Sci.* **2012**, *56*, 61–69. [CrossRef]

35. Blacquiere, T.; Altreuther, G.; Krieger, K.J. Evaluation of the Efficacy and Safety of Flumethrin 275 mg Bee-hive Strips (PolyVar Yellow®) against *Varroa destructor* in Naturally Infested Honey Bee Colonies in a Controlled Study. *Parasitol. Res.* **2017**, *116*, 109–122. [CrossRef]
36. Moro, A.; Mutinelli, F. Field evaluation of Maqs® and Api-Bioxal® for late summer control of Varroa mite infestation in Northeastern Italy. *J. Apic. Res.* **2018**, 53–61. [CrossRef]
37. Wilkinson, D.; Smith, G.C. A model of the mite parasite, *Varroa destructor*, on honeybees (*Apis mellifera*) to investigate parameters important to mite population growth. *Ecol. Model.* **2002**, *148*, 263–275. [CrossRef]