Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 2, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

THE SUSTAINABLE CONTROL OF VARROOSIS (*VARROA DESTRUCTOR*) BY TREATMENT OF CAPPED HONEYBEE BROOD USING ORGANIC VOLATILE ACIDS AND INNOVATIVE PROCEDURES

Adrian SICEANU, Eliza CĂUIA, Gabriela- Oana VIȘAN, Dumitru CĂUIA

Department of Honeybee Genetics and Breeding, Institute for Beekeeping Research and Development, 42 Ficusului Blvd, District 1, Bucharest, Romania

Corresponding author email: eliza.cauia@icdapicultura.ro

Abstract

The varroa mite infestation is a serious cause of honeybee colony loss at a global level. The varroa mite population development in the honeybee colony is the result of its reproduction success and of some favouring factors. Its parasitism model, which rely on capped brood for reproduction, as well as the role as vector of viruses increase the negative impact on honeybee health. Thus, there is clearly a necessity to develop new treatment approaches to interrupt the mite's life cycle, especially before winter honeybee rearing in order to protect it. Except for the formic acid, the substances used today, which generally treat the whole colony, target only phoretic mites. Using the formic and acetic acids' rapid vaporization properties, two procedures were developed and tested for the treatment of capped brood. The results show a high effectiveness in the mortality of mites (90%-100%) in different experimental variants. The capped brood brushing with volatile organic acids represents a highly effective, cost efficient, organic and minimally invasive procedure. It could be applied any time during the active season to decrease the level of infestation before critical moments.

Key words*: brushing, capped brood, honeybee, organic, varroa.*

INTRODUCTION

The worldwide depopulation and mortality of honeybees' colonies in the past decades, caused by different factors, has been widely documented (Potts et al., 2010; Neumann and Carreck, 2010; vanEngelsdorp et al., 2009).
One of the main causes of these mortalities, varroosis, was also largely studied (Traynor et al., 2020; Noël et al., 2020; Nazzi and Le Conte, 2016; Piou et al., 2016; Le Conte et al., 2010), its control being the subject of different, complex strategies (Roth et al., 2020; Dieteman et al., 2012).
Being an important vector for viruses, especially for the deformed wing virus - DWV, (Roberts et al., 2020; Dubois et al., 2020, 2019; Barrosso-Arévalo et al., 2019; Dainat et al., 2012a) and in light of the new findings showing that this parasite feeds primarily on the fat body of honeybees (Ramsey et al., 2018), the negative impact increases substantially, especially on winter honeybees' longevity and immunity (Di Prisco et al., 2016; Annoscia et al., 2015; Francis et al., 2013; Nazzi et al., 2012).
The mite *Varroa destructor* (Acari: Varroidae) (Anderson and Trueman et al., 2000) was described for the first time as the ectoparasite of *Apis cerana*, a species which copes very well with this parasitosis by complex adaptive, naturally selected traits, one of them being the almost exclusive reproduction of the varroa mite in drone brood, (Lin et al., 2018; Beaurepaire et al., 2015; Rath, 1999; Koeniger et al., 1983).
In *Apis mellifera*, varroa mite reproduction takes place in both, drone and worker brood, but there is a preference for drone brood in its rearing period, when the mite population could be 8-10-times greater (Rosenkranz et al., 2010; Boot, 1995; Boot et al., 1995; Boot et al., 1993; Fuchs, 1990). Following the differences in the post-capping period, an average of 1.3 -1.45 new mated females are produced in worker brood and 2.2-2.6 in drone brood (Martin, 1994). The success of its reproduction depends highly on the number of the reproductive cycles per each mated female, with an average of 2-3 reproductive cycles (Donze et al. 1998; Martin & Kemp, 1997; Ruijter et al., 1987), as well as on the type of brood. In the drone brood

it is 95%, while in the worker brood it is 73% (DeGrandi-Hoffman & Curry, 2004).
As it is well known, the life cycle of the varroa mite includes a phoretic phase, visible on adult bees, and a reproductive phase, which takes place in the capped brood, where new generations of mites are reared. Studies show that, in the active season, up to 90% of the varroa mite population can be found within the brood (Rosenkranz et al., 2010). Thus, the reproductive phase of mites has a very important negative impact on honeybees' health as both mature and immature mites feed intensively on brood, affecting the nutritional status and the immunity, as well as transmitting the viruses. As result of this complex varroa-honeybee relationship, combined with seasonal particularities and re-infestation risks, the varroa mite population in a colony is a dynamic process, with different levels of infestation between colonies, regions and time periods (DeGrandi-Hoffman & Curry, 2004; Martin, 1998; Fries, 1994;) which trigger the treatment strategies.
Regarding the reproduction phase, the varroa mite foundress enters a cell just before it is capped, for example in a 0-24 hours interval in the case of honeybee worker brood, and an even longer interval in the drone brood (Donze et al. 1998; Ruijter et al., 1987).
In the post capping period, the honeybee metamorphosis with different undergoing processes such as spinning the cocoon, pupation, moulting or pigmentation takes place under this cap and usually pass unobserved (Snodgrass, 1956; Rembold et al., 1980). In the same situation is the reproductive phase of the varroa mite, which is totally protected by the capping barrier, with negative consequences on the honeybee's natural defending mechanisms, such as grooming or hygiene mechanisms, as well as on the treatments' effectiveness.
Studying the brood capping closely, one can observe the presence of the two layers: (1) the external wax layer, applied by worker honeybees in order to protect the larvae from falling down during the pupation process (Siceanu, 1996), and (2) the internal layer, which is represented by the cocoon tissue formed in the pupation process right after capping (Snodgrass, 1956; Rembold et al., 1980). The external surface of the capping made by wax, which has the color of the neighbouring comb cells as an economic strategy of the honeybee colony, is rough and has small openings visible through a stereomicroscope.
However, the internal surface is smooth and glossy-white, with a relative transparency, allowing the wax colour to be slightly visible (Figures 1 and 2).

Figure 1. The external view of the brood cap in worker brood. In the green background one can notice small openings in the irregular composition

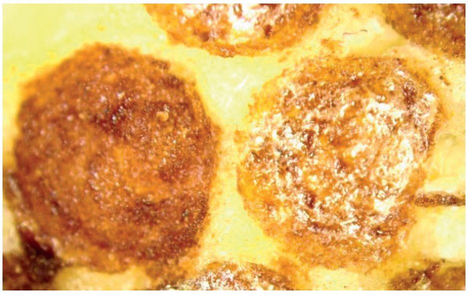


Figure 2. The internal view of the brood caps in worker brood. One can notice the white-shiny cocoon layer (right) and the wax layer after the cocoon was removed (left)

This porous, spongy-like structure of the honeybee brood cap, and the property of some organic substances (especially formic acid) to rapidly volatilise and pass through it, have recently led us to develop new procedures (Siceanu et al., 2019), for varroa mite control in capped brood. By their chemical properties (for example the pungent and irritating smell) (Formic acid-technical evaluation report, 2011), the highly volatile organic acids, like formic and acetic acids, affect the varroa mites through various mechanisms such as breathing inhibition (asphyxiation), disruption of the basic metabolic pathways (Rosenkranz et al.,

2010) and very likely by affecting the soft membranes (e.g., apoteles, intersegmental membranes) as well as by impairment of the sensory organs (e.g., pit organ), considering its chemosensing abilities (Nganso et al. 2020; Plettner et al., 2017).

Today, it is also well known that formic acid is the only substance that acts on brood when applied in the whole colony treatment, its effectiveness being very variable as many studies indicate: 41-95% (Calderón et al., 2010), 94.74% (Amrine & Noel, 2007), >60% (vanEngelsdorp et al., 2008). Some research even focused on brood treatment, separately by honeybee colony, for 1-2 hours, with very good results (up to 100% mite mortality) (Calis, 2001; Fries, 1991) and some practical information and applications were tried and recommended (Guido, 2018). The efficacity of formic acid on phoretic mites is also very variable (at least 40% and even over 95%), showing the importance of many factors involved, products or methods used (Pietrapaoli & Formato, 2019; Underwood & Currie, 2005, 2003; Elzen et al., 2004; Feldlaufer et al., 1997; Mutinelli et al., 1994). Most of these authors recommend the treatments of honeybee colonies with formic acid in long application (7-30 days) at the same time with monitoring the external temperature conditions in certain intervals which helps in vaporization control and reduction of the side-effects on bees. Unfortunately, the long duration of formic acid application can harm honeybees, queens, communications between individuals and the general development of the honeybee colony. These phenomena are highlighted in almost all the above-mentioned researches, as well as in practice. To overcome these problems, some new application methods were developed (Amrine & Noel, 2007; van Engelsdorp et al., 2008) to decrease the concentration and treatment duration, as the external temperature can be better predicted. The use of acetic acid in varroa mite control was also considered by researchers, but its effectiveness by whole colony treatment was lower than that of the formic acid (van Engelsdorp et al., 2008). To have a good effectiveness for varroa mite control, the use of highly volatile acids should be a very reasonable solution as they are also cost effective and organic substances.

Their use is allowed in varroa mite control in organic beekeeping in the European Union, as it is ruled in Council Regulation 834/2007, Regulation (EU) 2018/848 of the European Parliament.

Taking into account the negative effects of these substances on honeybees it is important to develop new methods of treatment, focusing only on capped brood (drone and worker), where the most part of varroa mite population exists in the active season. At the same time, this approach could be included in the sustainable strategies for varroa mite control which may be applied at any moment during the active season or at key moments, especially before rearing winter honeybees, in order to limit the natural development of the mite population, whose peak overlaps with this period.

Another advantage of limiting the treatment with volatile acids to capped brood combs is represented by a lower risk of honey contamination, having in view their hydrophilic properties and the presence of a higher content in honey, over the normal limits, following the conventional treatments.

In order to help the transfer of the volatile acids into brood cells by decreasing the treatment duration (from days or even hours to minutes), new procedures were developed and tested in our laboratory in recent years (artificial brood decapping, closed boxes using pression, brushing brood) (Siceanu et al., 2019). Following these preliminary researches, we focused on those treatment procedures that could be optimised and practically applied in beekeeping with very good results. Thus, the aim of the present study was to evaluate the effectiveness of two procedures for the capped brood treatment in very short time applications, on the mite (*Varroa destructor)* mortality inside the cells (the reproductive phase).

These procedures use highly volatile acids (formic and/or acetic acids) by (1) natural vaporization and saturation in closed space or by (2) capping brushing. If the first procedure - natural vaporization and saturation in closed space -- represents an improved procedure of the time-concentration parameters, following the researches published by Fries in 1991, and by Calis et al., in 2001 the second one - capped brood brushing -- represents a completely new procedure, firstly communicated and registered for patent by Siceanu et al. in 2019.

MATERIALS AND METHODS

1. Experimental design

To test the effectiveness of these treatment procedures, an experimental design was established and varroa mite mortality inside the capped brood, found in all the developmental stages, was assessed.

The applied procedures are based on:

(1) the air saturation with highly volatile acids by natural vaporization in a special airtight box, assuming that a high concentration will naturally and rapidly enter the capped cells, and

(2) brushing the capped brood combs directly with the highly volatile acids, using the natural properties of capping to absorb the substance and transfer it into cell for a short time interval.

The experiments were carried out in the 2018-2020 active seasons, in an experimental apiary (Băneasa 2) in the frame of Honeybee Genetic and Breeding Laboratory of the Institute for Beekeeping Research and Development - Bucharest (44°29′33″N 26°04′45″E). We included in the experimental apiary a total of 50 honeybee colonies of *Apis mellifera carpatica* subspecies, with young queens (2018, 2019), managed in Dadant hives on 10 frames. The experimental colonies have not been treated since 2018 in order to increase the level of varroa mite infestation for the 2019-2020 experiments. To increase the probability of having as much as possible a high infestation with varroa mite, for a better effectiveness in varroa mite counting, the procedure applications and the measurements were done from July 15th to August 30th, both in 2019 and 2020. At the same time and for the same reason, the donor colonies for capped brood combs were randomly selected from those with the highest level of infestation, being screened by natural mites that had fallen on the bottom boards. The experimental procedures were applied on honeybee capped brood combs, without adult bees (workers, drones, queen). To evaluate the impact of treatments on different categories of mites, the combs were generally selected to have brood of older ages (6-12 days post capping) in order to find as much as possible all the developmental stages of varroa mite.

A number of 10 combs was treated for each experimental variant according to the experimental design in Table 1 and the mite mortality evaluations were done under laboratory conditions.

As natural infestation of capped brood means, generally, varroa mites in a reproducing status and as they can be easily identified by the presence of white faecal deposits on the cell walls, a certain indicator of live mites (Dietemann et al., 2013; Büchler et al., 2017), control variants were not included to assess its natural mortality in the untreated capped brood. In some similar experiments (van Engelsdorp et al., 2008; Fries, 1991), the natural mortality of the varroa mite included in tests as control was extremely low.

Table 1. The experimental design for capped brood treatments by normal vaporization and by brushing the volatile acids

Experimental design and treatment variants	No. of treated combs	Concentration of active substance %	Quantity (ml.)
The experimental group to test the first procedure – The capped brood treatment, for different time intervals, in closed space, saturated with formic or acetic acid vapours by natural vaporization			
Formic acid treatment for 15 minutes (T1-FA 5')	10	85	100
Formic acid treatment for 10 minutes (T2-FA 10')	10	85	100
Formic acid treatment for 5 minutes (T3-FA 15')	10	85	100
Acetic acid treatment for 20 minutes (T4-AA 20')	10	99	100
The second experimental group to test the second procedure - The capped brood treatment by brushing with formic and acetic acids of different concentrations			
Brushing with formic acid 85% (T5-FAB 65%)	10	65	-
Brushing with formic acid 65% (T6-FAB 85%)	10	85	-
Brushing with acetic acid 99% (T7-AAB 80%)	10	80	-
Brushing with acetic acid 80% (T8-AAB 99%)	10	99	-
Brushing with a formula based on formic acid 65% and acetic acids 80% in different proportions* (T9-FAAB 65&80%)	10	65&80	-
*formic acid 65%, acetic acid 80%, plant extracts (*Ocimum basilicum, Thymi vulgaris, Mentha piperita, Mellisa officinalis*) and sugar in proportion of 6:2.5:1:0.5.			

Also, the experiments were designed to include different experimental variants grouped in the two procedures to test the specific variables (substance, time, concentration), so as to be able to perform comparisons, statistical analysis and data interpretation. The plants used in the extract are medicinal and aromatic plants, containing active substances recognized for positive effect on the honeybee digestive system and anti-repellent effect. The sugar role was to assure a good adherence of formula on the comb surface, to better maintain the formula substances in the porous structure of the cap. Thus, the formula based on formic and acetic acid (FAAB 65&80%), as well as some plant extracts and sugar, was specially created to decrease the concentrations of acids, to include the necessary active substances for the best efficacy on varroa mites' mortality, to have a good adherence, as well as to help attract honeybees after treatment to take care of the treated brood in a shorter period of time after treatment.

2. The procedures application.

2.1. The capped brood treatment, for different time intervals, in closed space, saturated with formic or acetic acid vapours by natural vaporization.

Before treatment (at least 10 minutes), an airtight box was prepared, by application of 100 ml formic acid of 85% concentration or acetic acid of 99% concentration on textile elements placed on lateral walls and on the inner cover, so as to sustain a rapid vaporization and air saturation inside the box. As a result of some measurements, the quantity of vaporised formic acid during the treatment of 4 combs, which is the frames capacity of the treatment box in our experiments (including all operations), was between 15 and 30 g at a volume of 33 dm^3.

In order to apply this procedure, irrespective of surface or presence of open brood or food, the worker honeybee capped brood combs to be treated were shaken and brushed off to eliminate the covering bees in the origin colony.

The combs were put into the airtight box, after saturation with formic acid by natural vaporization, they were treated for 5, 10 and 15 minutes. The treated combs were put back into the origin colonies until the next day when the mite mortality was assessed (Figure 3).

Figure 3. The application of treatment in closed space, saturated with organic, volatile acids vapours by natural evaporation

2.2. The application of treatment by brushing the capped brood surfaces with tested substances.

To apply this procedure, irrespective of the capped brood surface or the presence of open brood or food, the worker capped brood combs were shaken and brushed off to eliminate the covering bees in the origin colony. The brood combs were successively treated (brushed) with substances of different concentration or formula (Figure 4), depending on experimental variants and put into a ventilated box placed near the original hive (Figure 5).

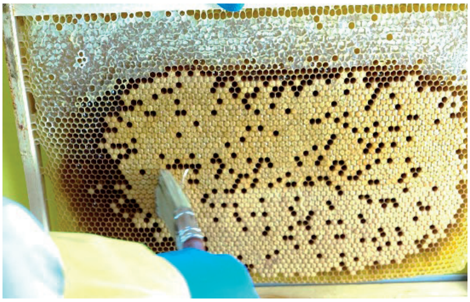


Figure 4. The application of treatment with volatile organic acids by brushing the capped brood

The honeycombs were held so that the treatment solution should not leak into the uncapped cells in which there could be eggs, brood larvae, honey or pollen bread, so as to avoid their contact with acids. To treat the combs with experimental substances, we used a paintbrush with medium stiffness bristles, about 4-10 cm wide. The treatment product was applied and brushed with a light press, to help the cap absorb the tested product. The surface of the capped brood was brushed so that all

cells with capped brood were also covered with the treatment substance. The brushing was done with left-right movements, to avoid the accumulation of drops on the lower edge of the uncapped cells and leakage inside them.
To carry out the treatment, the volatile acids were put into a special plastic box which is strongly fixed by the hive wall (Figure 5). The operation was repeated on all capped brood combs' surfaces from the experimental variants. The treated brood was immediately placed in a well-ventilated box hive type (e.g. frames transport box, swarm box, etc.) as shown in the figure 5. The box was covered with a board, so that the bees could not enter the space (to prevent robbing if there was a risk) and left for 10-15 minutes, during which time, most of the treatment substances evaporated inside and outside the cells.

Figure 5. The application of treatment with volatile organic acids by brushing the capped brood by a simplified variant, near the treated hive

The treated combs were not immediately returned to the colony because the amount of evaporated acids can harm the honeybees or queens in the honeybee colonies, especially in the first minutes. The direct contact of the testing acids with any individual (bees or queen) can kill them. For this reason, it is recommended to keep the treated combs after brushing in separately boxes for at least 10 minutes, depending on the treated surface, until the excess of substances is evaporated.
The treated combs were put back into the origin colonies until the next day when the mite mortality was assessed.
While using the treatment substances, it is mandatory to wear acid-resistant protective gloves, glasses and mask to prevent inhalation of acid vapours or direct contact.
To better understand this procedure, two scientific-technical video-films were developed and openly published (Siceanu et al., 2019; Siceanu, 2020).

3. The measurements on varroa mite mortality inside the capped cells

To give the treatment time for action, and to assess the impact of treatment on different categories of varroa mite which normally is found in the infested cells, the mortality was assessed on the day following the treatment (24 h). For each application procedure specific data about the treatment was registered (concentration, quantity, time), number of checked cells, number of infested cells as well as number of live and dead mites for each category. Thus, treated combs were taken out of the colony and the number of dead and alive varroa mites (including all individuals in a dying state) was assessed, using a stereomicroscope (Olympus SZ61) with 6.7X-45X magnification. To do these evaluations, the cells were opened with a tweezer, cell by cell, in rows, following the standard protocol (Dietemann et al., 2013; Büchler et al., 2017) or in some cases using the artificial decapping method to uncap rapidly a larger portion of cells (Siceanu et al., 2018, 1996). As mentioned above, the infested cells were more easily identified by the presence and white aspect of mite dejection on the cell walls. Each pupa from the infested cells were taken out and carefully put on a slide to be inspected. All the categories of varroa mites that were found and their state (dead or alive) were registered. In the same manner, the emptied cells were inspected. The varroa mites counting was assigned to the following different categories of mites according to their aspect: foundress females (FF), adult males (AM), protonymphs - males and females (P), deutonymphs - females (D), and adult daughters (AD) as shown in figure 6. The adult mites and immature stages (eggs, larvae, protonymphs, deutonymphs) present a sexual dimorphism and a gradual sclerotization of the exoskeleton which help their identification.

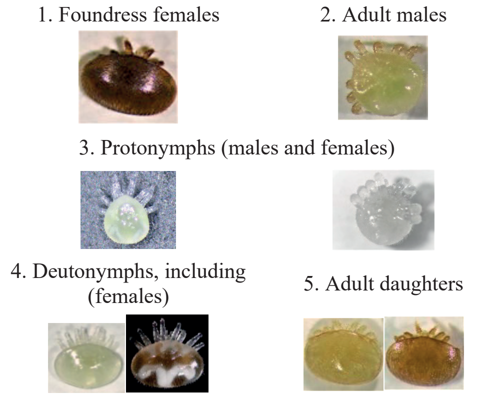


Figure 6. The aspect of different stages of varroa mite development in capped brood (6.7X-45X, stereomicroscope - Olympus SZ 61). Photos© Institute for Beekeeping Research and Development, Bucharest, Honey bee Genetics and Breeding Laboratory

As it is very difficult, confusing and time consuming to distinguish between protonymphs/deutonymphs of males when compared with protonymphs of females, these stages were included into protonymphs category of males and females, and from the treatment perspective they can be similarly affected as they are individuals of similar size with an unsclerotized exoskeleton.

Deutonymphs received a special attention as their immobile phase (which last 48h) (Dietemann et al., 2013), can be assigned to death category, the live individuals presenting an internal specific motility which can be noticed by their transparency. To notice these details, the deutonymphs were placed in a good position and light at a 45X magnification.

To perform statistical analyses on the obtained data, the tests for outlier's data identification (Grubbs test) and normal data distributions (Anderson Darling test) were firstly applied. To apply different statistical tests in order to assess the statistical significance threshold of different treatments' effectiveness, we used a Bartlett test for the variances' homogeneity, calculated in R software followed by specific tests to check the averages' homogeneity assumptions (Free software for statistical analysis). Thus, the homogeneity of the averages within each experimental group was analysed by a Welch's ANOVA test for unequal variance followed by a Games Howell post-hoc test in the frame of the first experimental group, and an ANOVA test followed by a Tukey post-hoc test for equal variance in the second experimental group. Data were calculated in Excel Office 2016 worksheets completed by XRealStats and Sigma XL modules, according to the statistical analysis guidelines presented in the literature (Sandu, 1995; Pirk et al., 2013). Additionally, a set of boxplots histograms on different treatments and categories of mites in the frame of the two groups of treatments were presented. It is important to mention that the percentage of varroa mite mortality 24 hours later, following the treatment application, was the response variable in all the statistical analyses.

RESULTS AND DISCUSSIONS

The obtained results regarding the average of varroa mite mortality in the cells (%), assessed at 24 hours after treatments application, in different treatments, are shown in tables 2, 3, and 4. The results were obtained by evaluating an average of 26.4 single or multiple infested cells per comb, out of 139.3 checked cells per comb in average, per total experiment. The general infestation level of brood combs on average was 19.5% (Table 3). According to these data, a high percentage of varroa mortality (>85%) was registered in more treatments performed by the two types of procedures: FA 10 min, FA 15 min, FAB 65%, FAB 85%, AAB 99%, and FAAB 65&80%. Analysing the averages, in the first experimental group (T1-T4), the best effectiveness of brood treatment (Ave. = 97.96%, St err. ± 0.56) was registered after keeping the capped brood combs in the saturated space with formic acid vapours for 15 minutes. A lower effectiveness (Ave. = 85.74%, St err. ± 1.89) was registered at a 10 minutes interval, while a low effectiveness (Ave. = 26.22%, St err. ± 1.44) was registered after 5 minutes of treatment. These data show an increasing effectiveness of the formic acid combating the varroa mite in a saturated space, in a certain time interval (5-15 minutes), with maximum effectiveness at 15 minutes treatment. The effectiveness of acetic acid 99% (Ave = 68.24%, St err. ± 1.27) when used to saturate a treatment space for 20 minutes was lower than that of the formic acid used for 10 minutes.

In the second experimental group (T5-T9) regarding the brushing of capped brood with volatile acids of different concentrations, a high effectiveness (over 90%) of treatments on varroa mite mortality inside the cells was registered in the experimental variants in which formic acid was used: FAB 65% (Ave. = 90.48%, St err. ± 1.29), FAB 85% (Ave. = 92.64%, St err. ± 1.38), and FAAB 65&80% (Ave. = 96.36%, St err. ± 0.84). Acetic acid of 99% and 80%, when used alone in brood brushing, showed a lower effectiveness (AAB 99%: Ave. = 89.68%, St err. ± 0.89, respectively AAB 99%: Ave. = 74.46%, St err. ± 1.88), but a better one than in the treatment in saturated box (AA 20'). For a better overview, the results of each experimental variant were plotted in figure 7, highlighting the quartiles repartition and averages of varroa mite mortality as percentage. Thus, one can easily remark the best treatments, also by values repartition on quartiles (75th, 50th and 25th) and overall average of each treatment.

Table 2. The varroa mite mortality percentage in average per each comb, in different experimental variants

Treated brood combs	*The 1st experimental group*				*The 2nd experimental group*				
	T1 FA 5'	**T2 FA 10'**	**T3 FA 15'**	**T4 AA 20'**	**T5 FAB 65%**	**T6 FAB 85%**	**T7 AAB 80%**	**T8 AAB 99%**	**T9 FAAB 65&80%**
C1	22.54	80.90	94.00	64.79	89.63	90.85	81.03	90.91	100.00
C2	32.65	78.43	98.08	64.29	88.27	95.83	82.76	91.85	93.33
C3	29.23	90.14	96.97	72.22	90.00	85.99	70.23	88.71	100.00
C4	25.53	86.61	99.07	71.43	96.10	92.12	78.70	90.72	98.55
C5	17.46	80.43	100.00	76.12	96.23	97.45	75.84	95.05	97.50
C6	26.09	86.67	96.77	68.14	88.24	95.92	66.67	90.00	92.55
C7	27.85	87.37	98.89	65.31	83.33	86.73	67.42	87.39	96.15
C8	22.22	92.50	97.83	67.09	87.95	89.11	78.38	89.47	96.05
C9	27.37	95.65	97.96	63.95	90.43	94.25	70.69	84.42	95.45
C10	31.25	78.69	100.00	69.09	94.64	98.17	72.86	88.30	94.00
Ave.	**26.22**	**85.74**	**97.96**	**68.24**	**90.48**	**92.64**	**74.46**	**89.68**	**96.36**
St. Err. ±	**1.44**	**1.89**	**0.56**	**1.27**	**1.29**	**1.38**	**1.80**	**0.89**	**0.84**

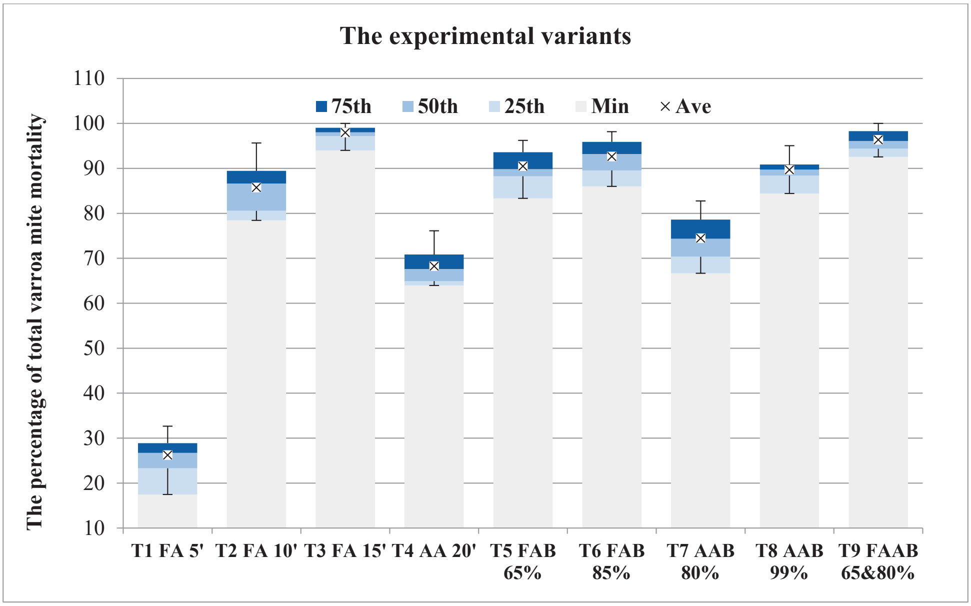


Figure 7. A box plot presentation of varroa mite mortality data (%) in capped brood treated with formic and acetic acids by experimentally tested procedures

Table 3. The obtained results regarding the number of checked cells, infested cells, varroa mites and the average of mortality on each treatment

Treatments	Number of combs	No. of checked cells	No. of evaluated infested cells	Infestation level %	The varroa mite's evaluation		
					Total (dead & alive) (T)	Dead (D)	Mortality % (M)
T1 - FA 5 min.	10	1356	178	13.13	606	158	26.07
T2 - FA 10 min.	10	1866	230	12.33	734	633	86.24
T3 - FA 15 min.	10	1228	168	13.68	763	749	98.17
T4 - AA 20 min.	10	1164	232	19.93	826	560	67.80
T5 - FAB 65%	10	1548	415	26.81	1609	1457	90.55
T6 - FAB 85%	10	1308	420	32.11	1632	1499	91.85
T7 - AAB 80%	10	1394	251	18.01	1189	890	74.85
T8 - AAB 99%	10	861	221	25.67	931	837	89.90
T9 - FAAB 65&80	10	1824	259	14.20	1030	988	95.92
Total	90	12549	2374	18.92	9320	7771	-
Ave.	10	1394.3	263.8	19.5	1035.6	863.4	83.38
St. Err. ± T1-T4	-	*89.85*	*9.52*	*0.98*	-	-	*15.79*
St. Err. ± T5-T9	-	*74.44*	*26.62*	*1.47*	-	-	*3.60*

Table 4. The obtained results regarding the number of varroa mites found in brood (total and dead) as well as its mortality in average (%) on different categories of mites and each treatment

Treatments	***The number of varroa mites found in brood and its mortality on different categories after treatments (at 24 h)***														
	Foundress			**Males**			**Protonymphs**			**Deutonymphs**			**Daughters**		
	T	**D**	**M %**	**T**	**D**	**M %**	**T**	**D**	**M %**	**T**	**D**	**M %**	**T**	**D**	**M %**
T1 FA 5 min.	188	37	**19.6**	82	26	**31.7**	147	42	**28.5**	115	32	**27.8**	74	21	**28.3**
T2 FA 10 min.	235	198	**84.2**	90	80	**88.8**	164	145	**88.4**	151	130	**86.0**	94	80	**85.1**
T3 FA 15 min.	168	164	**97.6**	84	82	**97.6**	215	215	**100.0**	198	193	**97.4**	98	95	**96.9**
T4 AA 20 min.	258	101	**39.1**	93	81	**87.1**	137	124	**90.5**	172	152	**88.3**	166	102	**61.4**
T5 FAB 65%	474	428	**90.3**	223	206	**92.3**	238	230	**96.6**	441	390	**88.4**	233	203	**87.1**
T6 FAB 85%	486	454	**93.4**	215	205	**95.3**	211	209	**99.0**	461	392	**85.0**	259	239	**92.2**
T7 AAB 80%	278	182	**65.4**	169	108	**63.9**	222	196	**88.2**	289	229	**79.2**	231	175	**75.7**
T8 AAB 99%	310	277	**89.3**	145	131	**90.3**	184	175	**95.1**	175	149	**85.1**	117	105	**89.74**
T9 FAAB 65&80	274	261	**95.2**	88	80	**90.9**	297	297	**100.0**	257	240	**93.3**	114	110	**96.4**
Total	**2671**	**2102**	**-**	**1189**	**999**	**-**	**1815**	**1633**	**-**	**2259**	**1907**	**-**	**1386**	**1130**	**-**
Ave.	**296.8**	**233.6**	**78.7**	**132.1**	**111.0**	**84.0**	**201.7**	**181.4**	**89.9**	**251.0**	**211.9**	**84.4**	**154.0**	**125.6**	**81.5**
St. Err. ± T1-T4	-	-	*18.4*	-	-	*15.0*	-	-	*16.3*	-	-	*15.8*	-	-	*15.1*
St. Err. ± T5-T9	-	-	*5.4*	-	-	*5.7*	-	-	*2.0*	-	-	*2.3*	-	-	*3.4*

In order to perform statistical analyses, the data were checked out for outliers' values, using the Grubbs test in Excel Office 2016 worksheets, checked out also by XRealStats software, the obtained results showing the lack of these data. Further on, the data normality was checked out using the Anderson-Darling test performed in Excel Office 2016 completed by Sigma XL module. The all obtained p-values were greater than the level of confidence (α=0.05) which validate the assumption that the data sampled are from a normal distribution.

To establish the homogeneity of variances of the tested samples, in a normal distribution of data, a Bartlett test performed in R software was performed for equal samples, all treatments and by groups of treatments. The results are presented in the table 5.

Table 5. The results on variances homogeneity – Bartlett test, equal samples

Treatments	**Bartlett's K-squared**	df	p-value	X^2 critic α=0.05	The results
T1-T9 (all treatments)	17.618	8	0.02428	15.51	unequal variance
T1-T4 (the 1st group of treatments)	10.543	3	0.01447	7.81	unequal variance
T5-T9 (the 2nd group of treatments)	6.8424	4	0.1445	9.49	equal variance

The obtained values and their probability show a heterogenic variance in the tested treatments which is generated by the first group of treatments, as by subsequently testing an unequal variance in the first group of treatments (K-squared > X2 critic, at α=0.05) and an equal variance in the second group of treatments was found.

To continue with the statistical analysis on the first group of treatments, a Welch's ANOVA test assuming unequal variance was applied to establish if the differences would be identified also concerning the treatments' averages.

Table 6. The Welch's ANOVA test of averages assuming unequal variances for the 1st group of treatments T1-T4

Welch's ANOVA test	Numerator df	Denominator df	F-calc.	Probability level
Between Groups	3	17.87	735.4	6.59E-19
F-critic (df 3; 18; α= 0.05) =3.16 F-critic (df 3; 18; α= 0.001) = 8.49 The result. F calc> F crit. The null hypothesis of equal averages is rejected				

The summarised results in the table 6 show highly significant differences between the averages of the 1st group of treatment.

As a result, a Games-Howell post-hoc test was applied further on to establish the statistical significance of differences between the averages of treatments, grouped two by two. The results are presented in table 7.

Table 7. The pair-wise comparison assuming unequal variances and equal samples (Games-Howell post-hoc test) for the first group of treatments T1-T4 (XRealStats)

Games-Howell test		Ave. diff.	q-calc.	df	q-crit α=0.05	p-val.
T1 FA 5'	T2 FA 10'	59.5	35.4	17	4.02	1.14E-13
T1 FA 5'	T3 FA 15'	71.7	65.6	12	4.20	-4.4E-13
T1 FA 5'	T4 AA 20'	42.0	30.9	18	4.00	1.66E-13
T2 FA 10'	T3 FA 15'	12.2	8.7	11	4.26	0.00039
T2 FA 10'	T4 AA 20'	17.5	10.8	16	4.05	5.72E-06
T3 FA 15'	T4 AA 20'	29.7	30.2	12	4.2	1.96E-10

As it can be easily noticed, there are highly significant differences between all treatments when compared two by two, highlighted by the pairwise average difference where q-calculated is higher than q-critic at a confidence level α=0.05. The lowest difference can be remarked between the 10 and 15 minutes treatments when formic acid was used.

Table 8. The results on averages' homogeneity (ANOVA single factor test), used for test the equal samples and equal variances for the 2nd group of treatments T5-T9

ANOVA single factor test					
Source of Variation	*SS*	*df*	*MS*	*F calc.*	*P-value*
Between treatments	2811.7	4	702.95	42.19	1.11E-14
Within treatments	749.73	45	16.66	-	-
Total	3561.5	49	-	-	-
F-critic (df 4; 45; α= 0.05) =2.61 F-critic (df 4; 45; α=0.001) = 5.70 F calc > F crit. The null hypothesis of equal averages is rejected					

The results of ANOVA single factor test, presented in table 8, show highly significant differences between all treatments as F calculated is higher than F critic (α = 0.001).

To statistically compare the treatments in the second group of treatments we used a one-way ANOVA test followed by a Tukey post-hoc test. Comparing the different brushing treatments by Tukey post-hoc test, to determine if at least one group of averages is different from the others, the following results (Table 9) were obtained:

Table 9. The pair-wise comparison assuming equal variances and equal samples (Tukey post-hoc test), for the second group of treatments T5-T9

Tukey test	T5-T9	T5-FAB 65%	T6-FAB 85%	T7-AAB 80%	T8-AAB 99%	T9-FAAB 65&80 %
T5-T9	Ave.	**90.48**	**92.64**	**74.46**	**89.68**	**96.36**
T5-FAB 65%	**90.48**	0	*2.16 (NS*)*	*-16.02 (HS)*	*-0.80 (NS)*	*5.88 (S)*
T6-FAB 85%	**92.64**	0.761	0	*-18.18 (HS)*	*-2.96 (NS)*	*3.72 (NS)*
T7-AAB 80%	**74.46**	2.58E-10	5.84E-12	0	*15.22 (HS)*	*21.90 (HS)*
T8-AAB 99%	**89.68**	0.992	0.491	1.09E-09	0	*6.68 (HS)*
T9-FAAB 65&80%	**96.36**	0.019	0.265	2.46E-14	0.005	0
w-critic (tab) = q (df 5; 45; α=0.05) = 5.21 w-critic (tab) = q (df 5; 45; α=0.01) = 6.36						
**NS – Non-significant differences; S - Significant differences; HS - Highly significant differences.*						

This statistical test shows us that the varroa mite mortality registered non-significant differences (NS, w calculated < w critic, at $\alpha = 0.01$) between the following brushing treatments:

- formic acid 85% and formic acid 65% concentration;
- formic acid 85% and formula based on formic and acetic acid (65&80%);
- formic acid 85% and acetic acid 99%;
- formic acid 65% and acetic acid 99%.

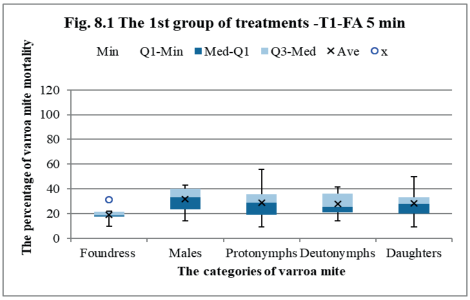
Comparing the treatments based on formic acid 65% with the formulation based on formic and acetic acids we registered significant differences (S) in varroa mortality at the level of confidence $\alpha = 0.05$, but no differences at $\alpha = 0.01$.

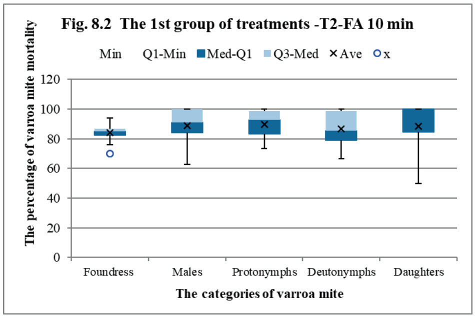
Highly significant differences in varroa mite mortality were found when the treatment formula was compared with acetic acids-based treatments, but important differences were found also between the two acetic acid-based treatments.

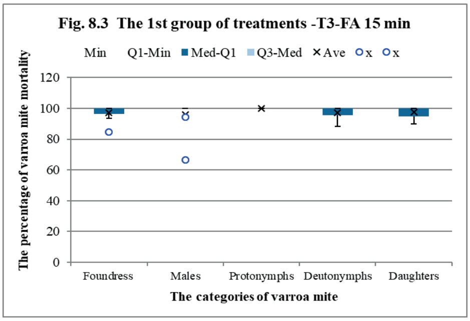
Highly significant differences were found also when acetic acid 99% was compared with formula based on formic and acetic acid, but at a lower level (w = 6.68, w calc at α at 0.01 = 6.36).

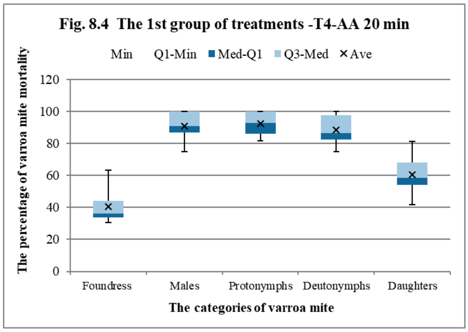
Regarding the different categories of varroa mite mortality in the brood cells (at 24 h) following the two procedures of treatment, the results on their mortality and standard error (±) for each treatment are presented in table S2.

For a better image of the data obtained on each treatment (n = 10 combs), box plots with quartiles, medians and averages as well as their limits of variation are presented in Figures 8.1 - 8.9.









Figures 8.1-8.4. Boxplots on different categories of varroa mite mortality in honeybee brood treated with formic and acetic acids by natural vaporization in closed space procedure

These figures show that in the 1st group of treatments, the formic acid act almost equal on different varroa categories, but the treatment duration is very important on the mortality level, while acetic acid act better on immature and unsclerotised varroa individuals. In the 2nd group of treatments one can notice that there is a better and more similar effectiveness on all varroa categories in using both active substances (formic and acetic acid) with lower values when using acetic acid alone and in lower concentration.

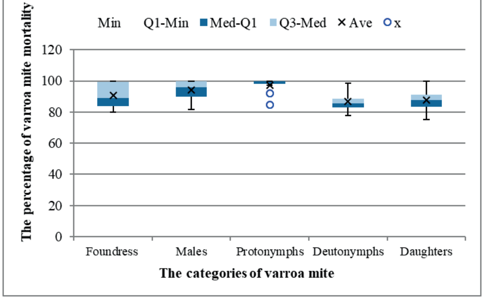


Fig. 8.5 The 2nd group of treatments -T5-FAB 65%

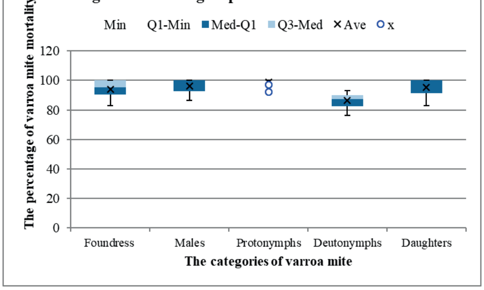


Fig. 8.6 The 2nd group of treatments -T6-FAB 85%

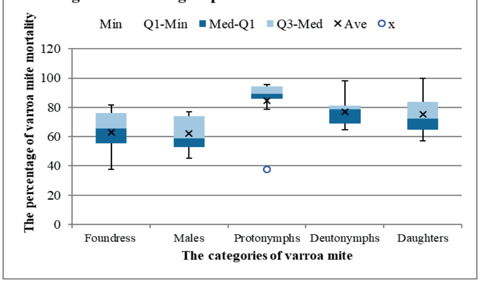


Fig. 8.7 The 2nd group of treatments -T7-AAB 80%

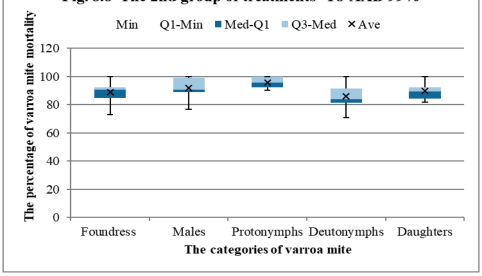


Fig. 8.8 The 2nd group of treatments -T8-AAB 99%

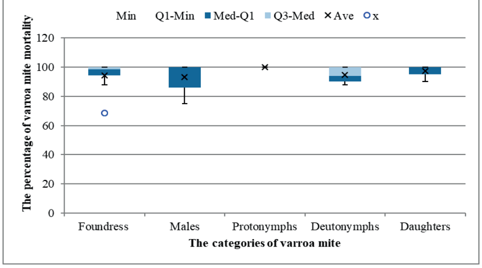


Fig. 8.9 The 2nd group of treatments -T9-FAAB 65&80%

Figures 8.5-8.9. Boxplots on different categories of varroa mite mortality in honeybee brood treated with formic and acetic acids by brushing procedure

The results we have obtained validate the hypothesis that the new tested treatment procedures are very effective (up to 100%) in treating *Varroa destructor* mite in the capped brood of the honeybee colonies, in short applications (minutes), severely interrupting the reproductive phase of varroa. However, the heterogenic variances and averages in the 1st group of treatments shows that the time parameter as well as the different volatilization properties of the two substances are very important in performing capped brood treatments in acid-saturated spaces, influencing the percentage of varroa mite mortality in the capped brood. Thus, the obtained results show us the importance of a minimal treatment duration, for acid molecules to penetrate the caps and make contact with the different categories of mites to have an immediate high mortality. This experiment shows us that, when the formic acid is used, it is important to keep the combs in the saturated boxes for a minimum of 10 minutes to have at least an 85% immediate mortality of varroa mite inside cells. In the second group of treatments, all the experimental brushing treatments having formic acid in their composition registered very good results on mortality of varroa mites. The best effectiveness was obtained with the formic acid of an 85% concentration or when the formula based on formic and acetic acid was used, but insignificant differences were registered between all treatments based on formic acid (65%, 85% and formula). Good results (on average an 89% mortality) were registered also when the acetic acid of 99% concentration was used and insignificant differences were found when it was compared with the formic acid 65% and 85%. The obtained results are better in the case of brushing procedures as once the capping is imbued, a part of the substance will immediately penetrate the cap and will fill the space of cells. As in the first group of treatments, the formic acid used by brushing procedure was proved to be more effective than acetic acid in order to obtain an immediate mortality, evaluated at 24 h after treatments.

According to the mortality level of different categories of varroa mite, the obtained results in the first group of treatments, where acid concentration varies with the treatment time (minutes) and the substance used (formic acid as compared with acetic acid), one can notice that adult females are the most resistant category to the treatment, especially when acetic acid is used, while the immature mites (protonymphs and deutonymphs) are more sensitive, especially protonymphs. This sensitivity depends most probably on the level of vapours (acid concentration) entering the cells and sclerotization degree of their body. The lack of sclerotization in immature stages of varroa brood is an important advantage in these treatments, especially if we want to decrease the time-concentration-dose parameters in the different treatment formula of current procedures.

Deutonymphs stages registered lower values because of the immobile phase which shows a greater resistance to volatiles, as in the case of the pupal stage in honeybees. This resistance can be noticed by observations done on the following stage - the freshly transformed daughters, which could be found live at the evaluation moment, on the next day after treatment. Being very effective in rapidly killing the mites, even the most resistant individuals (adult females), the use of formic and acetic acids in honeybee brood treatments can be considered safe for risks of resistance that these mites could develop, the organic volatile acids being recognized to pose minimal risks (Rosenkranz et al., 2010).

It is important also to mention different observations done during the evaluations:

- the most part of live varroa mites at the evaluation moment looked to be affected by these treatments, as a lower vitality was noticed during the evaluations.
- in some re-evaluations done two or three days after treatment, in the case of effective and very effective treatments (over 70% mortality), the adult females of varroa which remained alive were not capable to continue reproduction; they were found in a dying state, and the eggs were not present inside the cells anymore.

Consequently, from the varroa mite mortality evaluation perspective, we consider that the best moment for the evaluation of the treatments' effectiveness should be done at 2-3 days after the treatment, if there is no purpose to identify the different categories of mite progeny. After this period, the dead protonymphs and deutonymphs are in a decomposing stage and sometimes cannot be identified anymore, while the apparently live varroa mites on the first day after treatment as well as its reproduction activity can be clearly evaluated.

The life cycle of varroa mite would be seriously affected if the foundress is dead or in a dying state and its reproduction and offspring care (e.g., preparing the feeding site) will be affected, too. The same situation would be if the male is dead because the daughters, in case of survival (resulted from immobile deutonymphs) will not have been mated. Even if the viability of honeybee brood was not the purpose of this research, specific experiments being necessary, it was obvious to notice during the experiments that the pupal period was not affected by treatments, continuing its normal development. In these experiments, all the honeybees that emerged from the treated brood were found active and healthy, the hive population and activity being normal during the whole period of experiments. As we noticed, only the mobile stages found in the cocooning, pupation and emerging moments were found to be affected and only the individuals that passed through these stages in the interval of time that the brood was exposed to the substances, and these observations have already been documented even on a longer exposure – 1-2 hours (Calis, 2001; Fries, 1991). According to our observations as well as from older research (Siceanu, 1996), the honeybee pupal stages are more resistant to different factors than larval stages, especially when compared to open brood that requires regular feeding. In the capped brood period, only the nest temperature and humidity are important to the whole transformation from prepupa to adult honeybee. The scientific literature (Ruttner, 1980) shows that the honeybee brood, both larval and capped brood, if put outside the hive (not in sunlight) for a couple of hours or even more, is relative highly resistant. Thus, in the brood protection perspective, the brushing procedure can be considered superior to the treatment in a closed space as the volatile substances will come in contact only with the capped stages of honeybee brood and the mites inside cells, while in treatment boxes all brood, including larvae and eggs are treated and open brood is clearly affected. Having an immediate result and being targeted only on the capped brood frames, the effect of any external temperature and humidity do not influence the results and procedures' effectiveness as in the classical treatments with formic acids. More than this, by these new treatments we can avoid exposing the adult honeybees which are very sensitive to these substances, as their volatility is very high, increasing rapidly at high, external and nest temperatures.

Currently, at an international level, the treatment of capped brood with organic volatile acids is not practically used, the only method discussed in the literature and proposed in practice being the treatment in closed space (airtight box) for 1-2 hours (Guido, 2018; Calis, 2001; Fries, 1991). Shortening the time of treatment in boxes and developing totally new, minimally invasive and practical procedures such as brushing capped brood with effective volatile substance, would help beekeepers maintain a better control of varroosis. By enlarging the application period and choosing the key moments in the season, especially at the beginning of the season and before "winter bee" rearing, when the surface of capped brood is smaller, to minimize the workload or to combine with different local techniques whenever nest management is necessary (Siceanu, 2020), it is possible to increase substantially the benefit of this application and its effectiveness in combating varroa mite. For example, in the temperate season, the treatment may be done at any moment of the active season, when there is an intervention in the brood nest, even just before or during honey flows, as these substances do not contaminate the honey as well as all the other bee products, especially when applied by these procedures.

Actually, the majority of these treatments are done at the end of the summer season (e.g., August-October for the northern hemisphere, in temperate climate) when the honeybee colony population decreases and the mites' population increases and concentrates itself on the last brood and winter honeybee.

However, to drastically reduce the infestation level and disturb the population dynamic of the mite, the following key moments for applying these treatment procedures would be:

1. Apply early in the spring when there are small areas of capped brood, and the beekeeper performs some inspections or operations for reorganizing the nest (reduction or enlargement). Preferably, the treatment should be done before the beginning of drone rearing if the weather allows the interventions into the hive.
2. Apply when the artificial swarms are established using capped brood, usually with 1-3 frames of capped brood. This is an important treatment in order to give a clean start to the new colony, as usually a lot of varroa mites are taken out together with the capped brood.
3. Apply in the summer, just before the period of "winter bees" rearing, to produce healthy bees under a very low infestation. This can be done easily in the periods when there is a honey flow and the brood surfaces are reduced because the honeybees block the nest with honey, usually the beekeepers are forced to make room for egg laying to obtain bees for wintering.

Taking into consideration the 8-10-fold higher infestation rates of drone brood compared to worker brood, the treatment could be applied on all drone brood surfaces, which highly increases the effectiveness of overall treatment as well as the health of drones and reproduction biology.

In this concept of treatment, in order to kill also the phoretic varroa mites, two options could be available:

1) a classical treatment of honeybee colony with a rapid effect in the same period with brood treatment (e.g., the day before or after a brood treatment)

2) a second brood treatment with formic or acetic acids can be applied after 9 -12 days from the first treatment, a necessary interval of time to allow most part of phoretic mites (foundress females) enter the brood (before capping).

Decreasing the treatment duration and the concentration in active substances as well as the optimization of application procedures during normal inspections, are objectives for further investigations, in order to stimulate beekeepers to apply the capped brood treatment as well as to better protect the honeybee colony, brood and hive products.

Going further with the application possibilities, the new approach could be an effective treatment tool also in combating *Tropilaelaps* sp., taking into account the similarities regarding the reproductive and phoretic phases of these parasites, with a much shorter phoretic phase which contributes to the ineffectiveness of other treatments used in varroa mite control (Pettis et al., 2017; Raffique et al, 2012).

CONCLUSIONS

The two procedures using short time treatments with organic volatile acids are very effective in combating *Varroa destructor* mite in the reproductive phase, interrupting its life cycle.

According to the obtained data, a very high effectiveness of treatments (>90% mortality) was registered in four out of the nine experimental variants, at 24 h evaluation:

(1) the 15 minutes treatment of caped brood in saturated boxes with formic acid;
(2) the treatment of caped brood by brushing with formic acid of 85% concentration;
(3) the treatment of caped brood by brushing with formic acid of 65% concentration;
(4) the treatment of caped brood by brushing with a formula based on formic acid of 65% concentration and acetic acid of 80% concentration.

A good effectiveness (>85% mortality) was also registered in other two experimental variants:

(1) the 10 minutes treatment of caped brood in saturated boxes with formic acid;
(2) the treatment of caped brood by brushing with acetic acid of 99% concentration.

Both formic and acetic acids proved to be effective in saturated space, but their concentration is an important factor when used. For the first group of treatments, a 10 minutes treatment with formic acid in closed boxes should be sufficient, but further studies could better establish the optimum time-concentration variables. The new procedure of targeted capped brood treatment by brushing could be appreciated as better as compared with saturated space procedure as it does not affect the larval open brood, being a minimally invasive procedure especially with an optimised acid concentration formula. It valorises the natural property of caps to absorb and transfer the volatile organic substances into the cells, transforming its barrier role in a support for substances.

The effectiveness of new, optimal treatment formula for interrupting the life cycle of mite could be better evaluated after several days, when the reproductive success, live status and resistance of individuals can be better evaluated.

By applying the brood treatments in the key moments of the season, even earlier in the active season, and understanding the varroa mite-honeybee colony population dynamic, the level of infestation will decrease substantially, as well as the risks of colony collapsing in the inactive season.

By using the brood treatment and having in view the formic and acetic acids' property of rapid vaporization, the honey bee colony and by-products are not exposed to contamination substances, their impact being limited only to treated combs for a very short time period.

The present approach of brood treatment could open new ways to practical, flexible, organic and cost-efficient treatments in combating varroa mite in the world-wide beekeeping, in obtaining clean hive products for daily consume or apitherapeutic use, as well as in the multifactorial studies which aim to better study and explain the honeybee colony losses.

ACKNOWLEDGEMENTS

This research was funded by Ministry of Agriculture and Rural Development, The National Sectorial Plan ADER 2019-2022, project no. 12.1.1 /13.12.2019 and by Institute for Beekeeping

Research and Development Bucharest - Romanian Beekeepers' Association.
We are grateful to Prof. univ. Horia Grosu and Eng. Mircea-Cătălin Rotar for the important support in the statistical analysis of data.

REFERENCES

Amrine Jr., J.W., Noel, R., & Webb, D. (2007). Results of 50% formic acid fumigation of honey bee hives [*Apis mellifera ligustica* (Hymenoptera: Apidae)] to control varroa mites (Acari: Varroidae) in brood combs in Florida, U.S.A. *Internat. J. Acarol,* 33(2), 1–11. https://doi.org/10.1080/01647950708684508

Anderson, D.L., & Trueman, J.W. (2000). *Varroa jacobsoni* (Acari: Varroidae) is more than one species. *Exp Appl Acarol,* 24(3), 165–189. https://doi:10.1023/a:1006456720416.

Annoscia, D., Del Piccolo, F., Covre, F., & Nazzi. F. (2015). Mite infestation during development alters the in-hive behaviour of adult honeybees. *Apidologie*, *46,* 306–314. https://doi.org/10.1007/s13592-014-0323-0.

Barroso-Arévalo, S., Fernández-Carrión, E., Goyache, J., Molero, F., Puerta, F., & Sánchez-Vizcaíno, J.M. (2019). High load of deformed wing virus and *Varroa destructor* infestation are related to weakness of honey bee colonies in southern Spain. *Front. Microbiol.* https://doi:10.3389/fmicb.2019.01331

Beaurepaire, A.L., Truong, T.A., Fajardo, A.C., Dinh, T.Q., Cervancia, C., & Moritz, R.F.A. (2015). Host specificity in the honeybee parasitic mite, *Varroa spp.* in *Apis mellifera* and *Apis cerana*. *PLoS ONE* 10(8) https://doi.org/10.1371/journal.pone.0135103.

Boot, W.J. (1995). Invasion of Varroa mites into honey bee brood cells, *Thesis Wageningen* ISBN 90-5485-348-4. https://edepot.wur.nl/202208

Boot, W.J., van Baalen, M., & Sabelis, M.W. (1995). Why do Varroa mites invade worker brood cells of the honey bee despite lower reproductive success*?* *Behavioral Ecology and Sociobiology,* 36(4), 283–289. https://doi.10.1007/BF00165837.

Boot, W., Calis, J., & Beetsma, J. (1993). Invasion of *Varroa jacobsoni* into honey bee brood cells: a matter of chance or choice? *Journal of Apicultural Research,* 32(3-4), 167–174, https://doi:10.1080/00218839.1993.11101302

Büchler, R., Costa, C., Mondet, F., Kezic, N., & Kovacic, M. (2017). Screening for low varroa mite reproduction (SMR) and recapping in European honey bees. Available online: URL: https://dev.rescol.org/rnsbbweb/wp-content/uploads/2017/11/RNSBB_SMRrecapping_protocol_2017_09_11.pdf.

Calderón, R., Ramirez, M., & Ramirez, F. (2013) Control of varroa mites with formic acid and thymol in Africanised honey bee colonies, *The 43 International Apicultural Congress, Sept 29th – October 4th 2013, Kiew, Ukraine, Scientific Program and Abstracts*, 199–200.

Calis, J.N.M. (2001). Parasite-host interactions between the Varroa mite and the honey bee A contribution to sustainable Varroa control, Chapter 2. Control of varroa mites by combining trapping in honey bee worker brood with formic acid treatment of the capped brood outside the colony: putting knowledge on brood cell invasion into practice, *Thesis Wageningen Universiteit.* ISBN no. 99-5808-446-9, 33–46.

Dainat, B., Evans, J. D., Chen, Y. P., Gauthier, L., & Neumann, P., (2012a). Dead or alive: deformed wing virus and *Varroa destructor* reduce the life span of winter honeybees. *Applied Environmental Microbiology, 78,* 981–987. https://doi:10.1128/AEM.06537-11.

DeGrandi-Hoffman, G., & Curry, R. (2004) A mathematical model of Varroa mite (*Varroa destructor* Anderson and Trueman) and honeybee (*Apis mellifera* L.) population dynamics. *International Journal of Acarology, 30,* 259–274. https://doi:10.1080/01647950408684393.

Dietemann, V., Nazzi, F., Martin, S.J., Anderson, D., Locke, B., Delaplane, K.S., & Ellis, J.D. (2013) Standard methods for varroa research. The COLOSS BEEBOOK, Volume II: Standard methods for *Apis mellifera* pest and pathogen research. *Journal of Apicultural Research,* 52(1). https://doi:10.3896/IBRA.1.52.1.09.

Dietemann, V., Pflugfelder, J., Anderson, D., Charrière, J.-D., Chejanovsky, N., Dainat, B., De Miranda, J., Delaplane, K., Dillier, F.-X., Fuch, S., Gallmann, P., Gauthier, L., Imdorf, A., Koeniger, N., Kralj, J., Meikle, W., Pettis, J., Rosenkranz, P., Sammataro, D., Smith, D., Yañez, O., & Neumann, P. (2012). *Varroa destructor:* research avenues towards sustainable control, *Journal of Apicultural Research, 51(1),* 125–132. https://doi:10.3896/IBRA.1.51.1.15.

Di Prisco, G., Annoscia, D., Margiotta, M., Ferrara, R., Varricchio, P., Zanni, V., Caprio, E., Nazzi, F., & Pennacchio F. (2016). A mutualistic symbiosis between a parasitic mite and a pathogenic virus undermines honey bee immunity and health. *Proc. Natl. Acad. Sci*., *113,* 3203–3208. https://doi:10.1073/pnas.1523515113.

Donze, G., Fluri, P., & Imdorf, A. (1998). A look under the cap: the reproductive behaviour of varroa in the capped brood of the honey bee, Swiss Bee Research Centre. *American Bee Journal*, 138(7), 528–532.

Dubois, E., & Dalmon, A. (2020) Relations entre le virus des ailles déformées, l`acarien *Varroa destructor*, l`abeille mellifère, et leurs conséquences sur la sante des colonies, *La santé de l'abeille, 297,* 181–194.

Dubois, E., Dardouri, M., Schurr, F., Cougoule, N., Sircoulomb, F., & Thiéry, R. (2019) Outcomes of honeybee pupae inoculated with deformed wing virus genotypes A and B. *Apidologie*. https://doi:10.1007/s13592-019-00701-z.

Elzen, P.J., Westervelt, D., & Lucas, R. (2004) Formic acid treatment for control of *Varroa destructor* (Mesostigmata: Varroidae) and safety to *Apis mellifera* (Hymenoptera: Apidae) under southern United States conditions. *J. Econ. Entomol*., *97,* 1509–1512.

Feldlaufer, M., Pettis, J.S., Kochansky, J.P., & Shimanuki, H. (1997). A gel formulation of formic acid for the control of parasitic mites of honey bees. *American Bee Journal, 137*, 661–663.

Francis, R. M., Nielsen, S. L., & Kryger, P. (2013) Varroa-virus interaction in collapsing honey bee colonies. *PLoS One, 8.* https://doi:10.1371/journal.pone.0057540.

Formic Acid - Technical evaluation report (2011): *Retrieved from https://www.ams.usda.gov, https://www.ams.usda.gov/sites/default/files/media/Formic%20Acid%20TR.pdf.*

Free software for statistical analysis (R-project). *Retrieved from https://www.r-project.org/ and from https://cloud.r-project.org/*

Free software for statistical analysis (XRealStats). *Retrieved from https://www.real-statistics.com/free-download/real-statistics-resource-pack/*

Free software for statistical analysis (SigmaXL). *Retrieved from https://www.sigmaxl.com/SigmaXL%20Information.shtml.*

Fries, I., Camazine, S., & Sneyd, J. (1994). Population dynamics of *Varroa jacobsoni*: a model and review. *Bee World,* 75, 5–28.

Fries, I. (1991). Treatment of sealed honeybee brood with formic acid for control of *Varroa jacobsoni*. *American Bee Journal,* 131(5), 313–314.

Fuchs, S. (1990). Preference for drone brood by *Varroa jacobsoni* Oud. in colonies of *Apis mellifera carnica*, *Apidologie*, *21*, 193–199.

Guido, G. (2018) Formico Box - the gas chamber for varroa. *La santé de l'àbeille, 286,* 336–341. (in French).

Koeniger, N., Koeniger, G., & Delfinado-Bakerm, M. (1983). Observations on mites of the Asian honeybee species (*Apis cerana, Apis dorsata, Apis florea*). *Apidologie, 14,* 197–204.

Le Conte, Y., Ellis, M., & Ritter, W. (2010). Varroa mites and honey bee health: can Varroa explain part of the colony losses? *Apidologie,* 41 (3), 353–363. https://doi.org/10.1051/apido/2010017

Lin, Z., Qin, Y., Page P., Wang, S., Li, L., Wen, Z., Hu, F., Neumann, P., Zheng, H., & Dietemann, V. (2018). Reproduction of parasitic mites *Varroa destructor* in original and new honeybee hosts. *Ecology and Evolution, 8,* 2135–2145. https://doi.org/10.1002/ece3.3802.

Martin, S.J. (1998). A population model of the ectoparasitic mite *Varroa jacobsoni* in honey bee (*Apis mellifera*) colonies. *Ecological Modeling*, *109,* 267–281.

Martin, S.G., & Kemp, D. (1997). Average number of reproductive cycles performed by *Varroa jacobsoni* in honey bee (*Apis mellifera*) colonies. *Journal of Apicultural Research,* 36, 113–123. https://doi.10.1080/00218839.1997.11100937.

Martin, S.J. (1994). Ontogenesis of the mite *Varroa jacobsoni* Oud. in worker brood of the honeybee *Apis mellifera* L. under natural conditions. *Exp Appl Acarol., 18,* 87–100. https://doi.org/10.1007/BF00055033

Mutinelli, F., Cremasco, S., & Irsara, A. (1994). Formic acid in the control of varroatosis: A practical approach. *Journal of Veterinary Medicine Series B, 41*, 433–40. https://doi.org/10.1111/j.1439-0450.1994.tb00248.x

Nazzi, F., Brown, S. P., Annoscia, D., Del Piccolo, F., Di Prisco, G., Varricchio, P., Della Vedova, G., Cattonaro, F., Caprio, E., & Pennacchio, F. (2012). Synergistic parasite-pathogen interactions mediated by host immunity can drive the collapse of honeybee colonies. *PLoS Pathog*., *8.* https://doi: 10.1371/journal.ppat.1002735.

Nazzi, F., & Le Conte, Y. (2016). Ecology of *Varroa destructor*, the major ectoparasite of the western honey bee, *Apis mellifera*. *Annual Review of Entomology, 61*, 417–32. https://doi:10.1146/annurev-ento-010715-023731

Neumann, P., & Carreck, N., (2010). Honey bee colony losses*. Journal of Apicultural Research, 49*. https://doi:10.3896/IBRA.1.49.1.01.

Nganso, B.T., Mani, K., Altman, Y., Rafaeli, A., & Soroker, V. (2020). How crucial is the functional pit organ for the varroa mite? *Insects, 11,* 395. https://doi.org/10.3390/insects11060395

Noël, A., Le Conte, Y., & Mondet, F. (2020). *Varroa destructor*: how does it harm *Apis mellifera* honey bees and what can be done about it? *Emerging Topics in Life Sciences, 4*. https://doi:10.1042/ETLS20190125.

Patent Application (2021) Produs de uz veterinar în combaterea varroozei si procedure de aplicare (Product for veterinary use in combating varroosis and the application procedures) a2019/00483. *Buletin Oficial de Proprietate Industrială, BOPI no.2/2021, ISSN 2668-9006 ISSN-L 2668-9006, Retrieved from https://osim.ro/category/bopi/bopi-inventii/.*

Pettis, J.S., Rose, R., & Chaimanee, V. (2017). Chemical and cultural control of *Tropilaelaps mercedesae* mites in honeybee (*Apis mellifera*) colonies in Northern Thailand. *PLoS ONE,* 12(11). https://doi.org/10.1371/journal.pone.0188063.

Pietrapaoli, M., & Formato, G., (2019). Acaricide efficacy and honey bee toxicity of three new formic acid-based products to control *Varroa destructor*. *Journal of Apicultural Research,* 58(5), 824–830. https://doi.org/10.1080/00218839.2019.1656788

Piou, V., Tabart, J., Urrutia, V., Hemptinne, J.L., & Vétillard, A. (2016). Impact of the phoretic phase on reproduction and damage caused by *Varroa destructor* (Anderson and Trueman) to its host, the European honey bee (*Apis mellifera* L.). *PLoS ONE,* 11(4). https://doi.org/10.1371/journal.pone.0153482.

Pirk, C., de Miranda, J., Kramer, M., Murray, T., Nazzi, F., Shutler, D., Van der Steen, J., & van Dooremalen, C. (2013). Statistical guidelines for *Apis mellifera* research. *Journal of Apicultural Research, 52.* https://doi:10.3896/IBRA.1.52.4.13.

Plettner, E., Eliash, N., Singh, N.K., Pinnelli, G.R., & Soroker, V. (2017). The chemical ecology of host-parasite interaction as a target of *Varroa destructor* control agents, *Apidologie, 48,* 78–92.

Potts, S.G., Roberts, S.P.M., Dean, R., Marris, G., Brown, M.A., Jones, R., Neumann, P., & Settele, J. (2010). Declines of managed honey bees and beekeepers in Europe. *Journal of Apicultural*

Research, 49, 15–22. https://doi.org/10.3896/IBRA.1.49.1.02.
Raffique, M. K., Mahmood, R., Aslam, M., & Sarwar, G. (2012). Control of *Tropilaelaps clareae* mite by using formic acid and thymol in honey bee *Apis mellifera* L. colonies. *Pakistan J. Zool*., 44(4), 1129–1135.
Ramsey, S. D., Ochoa, R., Bauchan, G., Gulbronson, C., Mowery, J. D., Cohon, A., Lim, D., & vanEngelsdorp, D. (2018). *Varroa destructor* feeds primarily on honey bee fat body tissue and not hemolymph. *Proc. Natl. Acad. Sci. U.S.A.,* 116, 1792–1801. https://doi.org/10.1073/pnas.1818371116.
Rath, W. (1999). Co-adaptation of *Apis cerana* Fabr. and *Varroa jacobsoni* Oud. *Apidologie, 30,* 97–110, https://doi:10.1051/apido:19990202.
Rembold, H., Kremer, J.P., & Ulrich, G. (1980). Characterization of postembryonic developmental stages of the female castes of the honeybee, *Apis mellifera* L. *Apidologie*, 11(1), 29–38.
Roberts, J.M.K., Simbiken, N., Dale, C., Armstrong, J., & Anderson, D.L. (2020). Tolerance of honey bees to *Varroa* mite in the absence of deformed wing virus. *Viruses, 12*, 575. https://doi.org/10.3390/v12050575.
Rosenkranz, P., Aumeier, P., & Ziegelmann, B. (2010). Biology and control of *Varroa destructor*. *Journal of Invertebrate Pathology, 103,* 96–119. https://doi:10.1016/j.jip.2009.07.016.
Roth, M., Wilson, J., Tignor, K., & Gross, A. (2020). Biology and management of *Varroa destructor* (Mesostigmata: Varroidae) in *Apis mellifera* (Hymenoptera: Apidae) colonies. *Journal of Integrated Pest Management,* 11(1) 1–8. https://doi:10.1093/jipm/pmz036.
Ruijter, A. (1987). Reproduction of *Varroa jacobsoni* during successive brood cycles of the honeybee. *Apidologie,* 18(4), 321–326.
Ruttner, F. (1980). Queen Rearing, Chapter V, subchapters 1.2.1 -1.2.2, *Apimondia Publishing House Bucharest,* 86–88 (in Romanian).
Sandu, G. (1995). *Modele experimentale in zootehnie / Experimental models in animal breeding*, Bucharest, RO: Coral Sanivet Publishing House, 126–155.
Siceanu, A. (2020). A simplified procedure for capped brood brushing with organic volatile acids to be used in varroosis, *Retrieved from https://youtu.be/OmfUlEDY1Fg.*
Siceanu, A., Căuia, E., Vișan, G.O., & Căuia, D. (2019). Preliminary researches regarding the effectiveness of the formic acid treatment on varroa (*Varroa destructor*) found in the artificially decapped bee brood. *Journal of Agricultural Science and Technology A & B*, 9, 248–261, https://doi:10.17265/2161-6256/2019.04.005.
Siceanu, A., Căuia, E., Vișan, G.O., & Căuia, D. (2019). Preliminary researches regarding the efficacy of formic acid and acetic acid in the treatment of varroa (*Varroa destructor*) found in bee brood. The 15th Collos Conference Proceedings, Montreal. *Retrieved from https://coloss.org/event/coloss-conference-2019/.*
Siceanu, A., Căuia, E., Vișan, G.O., & Căuia, D. (2019). The optimisation of varroosis treatment methods with formic acid: a comparative approach 2018–2019. The 46th Apimondia Congress, 8–12 September 2019, Montreal, Abstract Book. 220.
Siceanu, A., Căuia, E., Vișan, G.O., & Căuia, D. (2019). A technical-scientific film: Research study on combating the *Varroa destructor* mite in capped brood of honeybees (*Apis mellifera*). *Retrieved from https://youtu.be/eptG6T4QnbA.*
Siceanu, A., Căuia, E., & Vișan, G. O. (2018). Preliminary protocol to identify the decapped-recapped cells by worker bees, in order to estimate the hygienic behaviour in connection with the capped brood.*Proceedings of Eurbee 8th Congress Apidology, Program and Abstract Book*, 177.
Siceanu, A. (1996, published online 2016). The artificial decapping of the honeybee brood in order to control the specific diseases. *Retrieved from https://www.youtube.com/watch?v=qHq2woncbN4.*
Siceanu, A. (1996). The artificial decapping of the honeybee brood for the control of the *Varroa jacobsoni* parasite. *Apiacta, 31,* 45–50.
Snodgrass, R.E. (1956). Anatomy of the honey bee, *Cornel University Press,* ISBN 1-904846-05-X, (renewed 2004).
Traynor, K.S., Mondet, F., Miranda, J.R., Techer, M., Kowallik, V., Oddie, M.A.Y., Chantawannakul, P., & McAfee, A. (2020) *Varroa destructor*: A Complex parasite, crippling honey bees worldwide, *Trends in Parasitology,* 36(7), 592-606, ISSN 1471–4922. https://doi.org/10.1016/j.pt.2020.04.004.
Underwood, R.M., & Currie, R.W. (2003). The effects of temperature and dose of formic acid on treatment efficacy against *Varroa destructor* (Acari: Varroidae): a parasite of *Apis mellifera* (Hymenoptera: Apidae). *Experimental and Applied Acaralogy, 29,* 303–313.
Underwood, R.M., & Currie, R.W. (2005). Effects of concentration and exposure time on treatment efficacy against varroa mite (Acari: Varroidae), during indoor winter fumigation of honeybees (Hymenoptera: Apidae), with formic acid. *Journal of Economic Entomology*, 98(6), 1802–1809. https://doi.10.1093/jee/98.6.1802 .
vanEngelsdorp, D., Evans, J.D., Saegerman, C., Mullin, C., Haubruge, E., Nguyen, B.K., Frazier, M., Frazier, J., Cox-Foster, D., Chen, Y., Underwood, R., Tarpy, D.R., & Pettis, J.S. (2009). Colony collapse disorder: a descriptive study. *PLoS One, 4.* https://doi:10.1371/journal.pone.0006481.
vanEngelsdorp, D., Underwood, R.M., & Cox-Foster, D.L. (2008). Short-term fumigation of honey bee (Hymenoptera: Apidae) colonies with formic and acetic acids for the control of *Varroa destructor* (Acari: Varroidae). *Journal of Economic Entomology,* 101(2), 256–264. 10.1603/0022-0493(2008)101[256:sfohbh]2.0.co;2

TECHNOLOGIES OF THE AGRO FOOD PRODUCTS PROCESSING