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Original article

Combination of thymol treatment (Apiguard®) and caging the queen technique to fight *Varroa destructor*

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Abstract – Guaranteeing high acaricide efficacy to control *Varroa destructor* is fundamental for colony survival. In this study, we verified the efficacy and impact of a commercial thymol-based veterinary product (Apiguard®) on colony honey bee populations when used alone or combined with the biotechnical method of caging honey bee queens to create an artificial brood interruption period in the colony. Apiguard® killed 76.1% of the mites while queen caging killed 40.6% of the mites. The combination of Apiguard® administration with queen caging killed 96.8% of the mites. Comparing bee numbers before and after treatment, Apiguard® treated colonies with caged queens had 48.7% fewer bees compared to before treatment, while Apiguard® alone reduced the number of adult bees by 13.6%. None of the treatments in the different groups resulted in elevated queen mortality.

Varroa destructor **/ thymol / Apiguard / queen caging / efficacy**

1. INTRODUCTION

The fight against *Varroa destructor* (*V. destructor*) (Anderson and Trueman 2000) continues to be one of the most difficult management aspects in apiculture worldwide (De Jong 1990; Sammataro *et al.* 2000). Considering the biology of this mite and its tendency to develop resistance to chemical compounds (Ritter and Roth 1988; Milani 1994; Lodesani *et al*. 1995; Milani 1999; Baxter *et al*. 2000; Della Vedova *et al*. 1997; Trouiller 1998; Elzen *et al*. 1999; Elzen and Westervelt 2002; Milani and Della Vedova 2002; Pettis 2004). eradication seems to be virtually impossible. Thus, it is important to verify and increase acaricide efficacy of existing products to keep infestation lower than the levels that impact colony survival.

"Soft" acaricides (Rosenkranz *et al.* 2010) like formic acid, oxalic acid, lactic acid and thymol present a low risk of residues and accumulation in bee products and do not lead to mite resistance (Imdorf *et al.* 1999; Rosenkranz *et al.* 2010). Formic acid is the only acaricide which is able to kill mites within sealed brood cells (Fries 1991).

Appendix I shows commercially available, ready-made preparations, including thymol-based ones, that are traded worldwide. The actives are frequently formulated within matrices (e.g. gel or vermiculite tablets or cellulose wafers) that allow their gradual and steady release (Mautz 1982; Mikityuk 1983; Lodesani *et al.* 1990; Mattila and Otis 1999; Mattila and Otis 2000; Mattila *et al.* 2000; Marinelli *et al.* 2001; Marinelli *et al.* 2008; El-Ghamdy 2002; Melathopoulos and Gates 2003; Baggio *et al.* 2004; Floris *et al.* 2004; Gregorc and Planinc 2005; Arculeo *et al.* 2006; Cebotari *et al.* 2006; Coffey 2007; Palmeri *et al.* 2007; Lodesani and Costa 2008; Loucif-Ayad *et al.* 2010).

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Thymol efficacy depends on the evaporation of the active principle within the hive, based on climatic temperatures and colony conditions (El-Ghamdy 2002; Lodesani and Costa 2008; Rosenkranz et al. 2010) and is ineffective on mites in their reproductive phase within brood cells.

According to Rosenkranz et al. (2010). biotechnical methods are sustainable approaches for *Varroa* treatment. A number of investigators attempted to identify efficient management techniques based on the biotechnical control of *V. destructor*. These methods include, among others: the removal of drone brood (Calderone 2005; Delaplane *et al.* 2005). heat treatment (Hoppe and Ritter 1987; Huang 2001) and the use of entomo-pathogenic fungi (Chandler *et al.* 2000; Shaw *et al.* 2002). The technique of caging the queen allows one to create an artificial brood interruption period in the colony; since mites rely on honey bee brood to reproduce, any break in the brood cycle would interrupt *V. destructor* population growth. Maul (1983) and Calis et al. (1999) experimented with temporary queen trapping in combination with the removal of sealed brood. Nanetti and Pietropaoli (Nanetti *et al.* 2012; Pietropaoli *et al.* 2012) coupled queen trapping with acaricides to increase the efficacy of the products.

This paper reports the results of our study to evaluate the impact of the biotechnical method of caging the queen, in combination with thymol treatment, on colony *Varroa* populations.

2. MATERIALS AND METHODS

During summer 2008 (August), we undertook field trials to evaluate the application of thymol (Apiguard®) alone or combined with queen caging to control *V. destructor* infestations. Concurrently, we also assessed the toxicity of these above mentioned treatments on the honey bees.

Apiguard® (Vita Europe Ltd, Basingstoke, Hants, United Kingdom - http://www.vita-europe.com/products/apiguard/#HowtouseApiguard), is a natural product patented as a slow-release gel containing thymol specifically designed for use in beehives. It is commercially available in aluminum trays containing 12.5g of thymol in 50g of gel. According to the summary of product characteristics, the tray has to be placed on top of brood frames and left in the hive for two weeks. Following this, it is replaced with a new tray that will be left in place for an additional 2 weeks. Moreover, according to the Vita Europe website indications, if the tray is almost empty after 10 days, it is possible to replace it with a second tray.

Queens were caged in VAR-CONTROL® cages (Api-Mo.Bru, Campodoro, Padova, Italy – http://www.apimobru.com/en/ppe/ppe.htm), which are plastic cages used to confine the queen that, at the same time, permit the access to worker bees that care for the queen (Figure 1). VAR-CONTROL® cages are 5 cm wide x 7.8 cm high and 3 cm deep. Once confined in the cage, the queen ceases to lay eggs throughout the caging period, thus limiting *V. destructor* reproduction in the honey bee brood. We located cages with the queens in the lower part of the frames to reduce their exposure to the thymol vapours originated from the tray placed on top of the frames (Figure 1).

The field trials were undertaken in two locations characterized by a temperate climate in the Latium region (central Italy). The two sites were 5 km north-east (Site-Apiary 1: Lat 41.550298; Long 12.983336) and 16 km north-west (Site-Apiary 2: Lat 41.433644; Long 12.836097) of Latina city respectively, 21 km from one another, in the same pedo-climatic area (Figure 2). According to Worldclim.org (Hijmans *et al.* 2005). temperatures (min, max and mean) and rainfall in August in the last 50 years (1950–2000) in the two areas were very similar: mean temperature was 23.3°C in Site-Apiary 1 and 23.5°C in Site-Apiary 2; minimum temperature was 16.8° C in Site-Apiary 1 and 17.9 °C in Site-Apiary2; maximum temperature was

Figure 1. VAR-CONTROL® cage, positioned in the lower part of the frame.

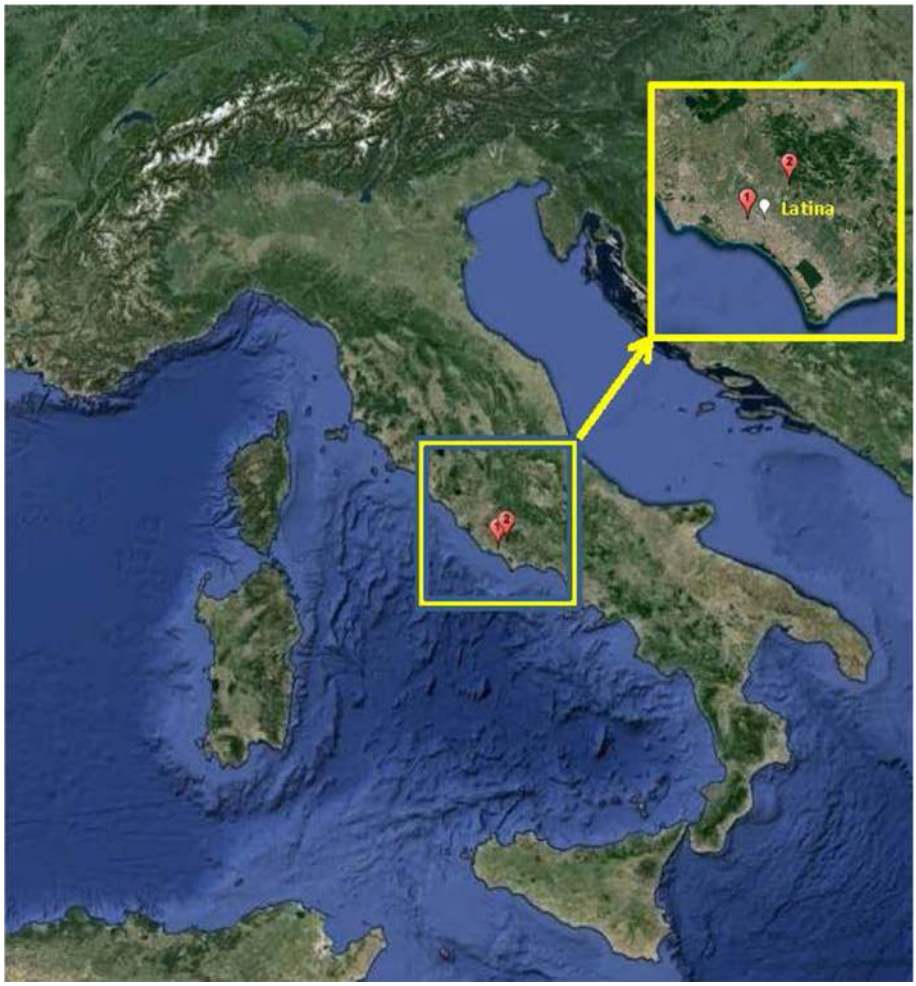


Figure 2. Location of the apiaries in Latina (Central Italy) where the field trials were conducted.

29.7°C in Site-Apiary 1 and 29.2°C in Site-Apiary 2; rainfall was 45 mm in Site-Apiary 1 and 38 mm in Site-Apiary 2. For this reason, considering that environmental conditions and management were the same in both apiaries, data of treated groups were combined as a unique sample.

In total, 46 honey bee colonies were monitored: 24 in Apiary 1 and 22 in Apiary 2. The 46 colonies were randomly divided into four different groups: (1) 10 colonies were treated with one tray of Apiguard® twice consecutively for a period of 10 days per tray ("Apiguard" group); (2) queens were caged in 12 colonies for 20 days using VAR-CONTROL® cages ("Queen caging" group); (3) 15 colonies were treated with Apiguard® as before and queens were caged for 20 days ("Apiguard® plus queen caging"); and (4) 9 hives were left untreated to understand natural mite mortality ("Control"). Colonies were housed in 10 frame Dadant-Blatt bee hives, had a similar strength and were free of any other symptomatic disease, except for varroatosis. The infestation levels between two apiaries and different treatment groups were similar. The infestation recorded in the groups ranged from 0.04 to 0.06 adult *Varroa* per bee. To verify the homogeneity of initial *Varroa* infestation of the selected colonies in the two apiaries, the natural mite fall was recorded for two weeks (Figure 3) before

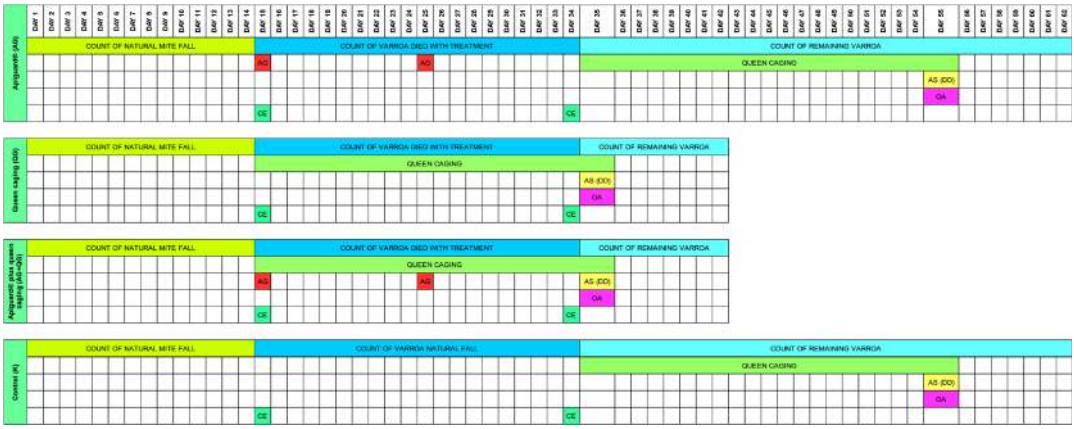


Figure 3. Gantt chart of the protocol followed for each treatment group. Treatment groups: "Apiguard" (1); "Queen caging" treatment (2); "Apiguard® plus queen caging" (3); "Control" (4). Key: AG=Apiguard® treatment; CE=colony strength estimation; AS (DD)=Apistan double dosage treatment; OA=Oxalic acid treatment.

starting the trials (Branco *et al.* 2006) and standardized against the estimated number of adult bees.

In our field trials we applied Apiguard® for 20 days (alone or combined with queen caging): 10 days of treatment with the first tray, followed by another 10 days with the second tray of the product (Figure 3). As also reported by Floris (Floris *et al.* 2004). we observed that the entire product was completely evaporated from the tray after 10 days of application.

Since drone brood was absent during summer, we evaluated mite fall over 21 days of queen caging, which is the time required for all workers to emerge (Figure 3).

Over the field trial period, mite fall was recorded every 3–4 days using sticky boards placed on the bottom board.

After the 20-day treatment with thymol, we evaluated the number of surviving mites by counting mite fall after the application for one week of a double dose (4 strips/hive) of Apistan® (tau-fluvalinate; Vita Europe Ltd, Basingstoke, Hants, United Kingdom) and a single dose of trickled oxalic acid solution in absence of brood. The absence of brood was already present in group 2 and 3, or obtained by caging the queen for 21 days in group 1 and 4 (Figure 3).

The oxalic acid solution administered consisted of 5 grams of oxalic acid dehydrate (Carlo Erba Reactifs SA, Chaussée du Vexin, BP 616, de Reuil, France) per hive in 50 mL of syrup (water and sucrose in a 1:1 ratio) and was applied at a rate of 5 mL of syrup for each area between combs occupied by bees.

The percentage of acaricide efficacy (AE) in each hive was evaluated using the following formula: $AE = \frac{V_T}{V_{(T+OA+APISTAN)}} * 100$, where V_T is the total number of mites killed with the treatment and $V_{(T+OA+APISTAN)}$ represents the total number of mites killed by the tested treatment, the oxalic acid and the Apistan® treatments (Dietemann *et al.* 2013).

Statistical analysis was performed to compare the efficacy of the treatments. The analysis only included colonies that had a level of infestation between 300 and 3.000 mites per colony, as indicated in the guideline on veterinary medicinal products controlling *V. destructor* parasitosis in bees (EMA 2008).

To determine the impact of Apiguard and queen caging on the number of adult honey bees in the treated hives, we estimated the colony populations (adult bees) at the beginning of the treatments (day 15) and on day 34 (Figure 3).

We visually estimated the number of bees observing frame sections covered by honey bees as proposed by Delaplane (Delaplane *et al.* 2013).

After the angular transformation of proportions, the Kruskal-Wallis test (Kruskal and Wallis 1952). followed by Mann–Whitney U test (Mann and Whitney 1947) with Bonferroni's correction when significant, was used to assess the difference of acaricidal efficacy and the difference in adult bees population survival. Data were reported as medians and 25th and 75th percentiles in

brackets. P-value <0.05 (two-tailed) was considered statistically significant in all analyses.

Data were analyzed by STATA/SE for Windows® Software (12.1 Version, Texas, USA).

Over the trial period, climatic temperatures (minimum, maximum and mean) were recorded daily to verify proper evaporation of the essential oil as thymol evaporation greatly increases with increasing temperature (Imdorf *et al.* 1999) and is most efficacious at a temperature range between 15°C and 35°C. In fact, at higher temperatures the product evaporation rate is very high and could cause absconding episodes in honey bees or harm the queens, while at lower temperatures the active substance may not evaporate sufficiently, resulting in low acaricide performance. Air humidity was not recorded, as thymol evaporation is not influenced by this parameter (Mikityuk 1983).

3. RESULTS

The acaricide efficacy (Figure 4) obtained with Apiguard® treatment alone was 76.1% (60.5%-86.3%), while queen caging alone resulted in 40.6% (30.2%-47.8%) efficacy. Apiguard® administration and queen caging undertaken together reduced mite populations by 96.8% (93.1%-98.9%), while natural mite mortality in the "Control" group was 5.2% (3.8%-8.5%). The

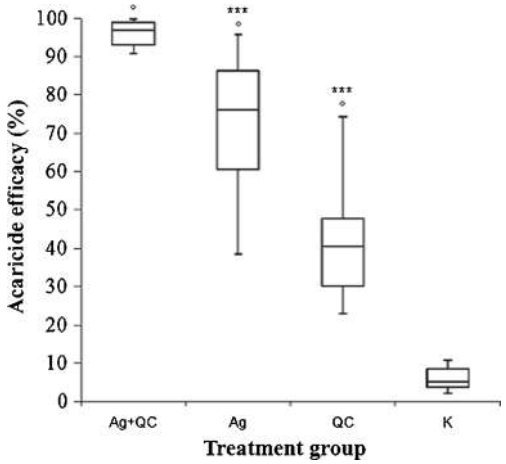


Figure 4. Box plot of acaricide efficacy (%) obtained for the four groups in the apiaries under study. (key: Ag = Apiguard®; QC = Queen Caging; Ag+QC = Apiguard® plus Queen Caging; K = Control). ° P <0.001 vs K. *** P <0.001 vs Ag+QC.

overall analysis of efficacy revealed that there was a difference of efficacy among treatments (P<0.001).

The acaricide effect of Apiguard® combined with queen caging was significantly greater than that produced by Apiguard® or queen caging alone (P <=0.001). There was no statistical difference in acaricide efficacy between the Apiguard® and queen caging alone groups (P =0.072).

The dynamics of mite fall registered are reported in Figure 5.

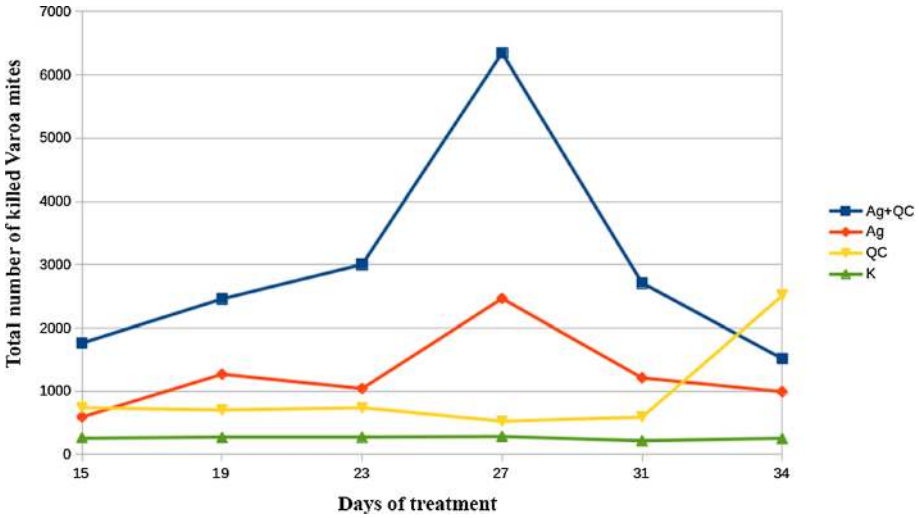
Table I shows the cumulative number of adult bees estimated in the four groups before and after the treatments. The percent survival was: 86.4% (ranging from 65.3% to 112.9%) in the "Apiguard®" group, 61.1% (ranging from 32.4% to 91.3%) in the "Queen caging" group, 51.3% (ranging from 34.8% to 67.3%) in the "Apiguard® plus queen caging" group, and 79.9% (ranging from 41.9% to 123.1%) in the "Control" group. The difference in adult bee population reduction between the "Apiguard® plus queen caging" and "Apiguard®" groups was statistically significant (P<0.001). Likewise, the difference in adult bee population reduction between the "Apiguard®" and "Queen caging" groups was statistically significant (P<0.001). There were no significant differences in the number of adult bees between the "Apiguard®" and "Control" groups (P =0.999) or between the "Apiguard® plus queen caging" and the "Queen caging" groups (P=0.999).

None of the treatments resulted in queen mortality in the four experimental groups.

The external temperatures in the two tested apiaries mostly remained within the optimum temperature range described for Apiguard® during the 20-days of treatment (Figure 6). In fact the mean temperature was 24.1°C and the maximum was never over 35°C. On six nights the minimum temperatures were lower than the ideal 15°, ranging between 13.5°C and 14.6°C.

4. DISCUSSION

Thymol (Apiguard®) treatment in conjunction with the queen caging technique resulted in higher acaricide performance (Figure 4). These results could be explained by the ability of thymol to kill



Figur 5. Total number of killed varroa mites in the four groups during the 20-days of Apiguard® treatment. (key: Ag = Apiguard®; QC = Queen Caging; Ag+QC = Apiguard® plus Queen Caging; K = Control).

the mites on the adult honey bees and its inability to kill them in the capped brood (Calderone 1999; Imdorf *et al.* 1999). Indeed, queen caging alone is able to reduce *Varroa* infestation in the colonies, probably because of an increase of the grooming activity of honey bees in absence of brood (Formato *et al.* 2008). The concurrent application of Apiguard® and queen caging increases the acaricide efficacy of both methods since mites are no longer able to enter the brood cells and can be killed by thymol, and reduce the variability of the varroacide efficacy among hives (Figure 4).

Mite fall dynamics shows that the greatest *Varroa* fall in the "Apiguard® plus queen caging" and in the "Apiguard®" groups is observed immediately after placing the 2nd tray in the hives. In contrast, the mite fall in the "Queen caging" group increased when almost all capped brood emerged. It would be interesting to determine how much the final acaricide efficacy of this group is enhanced when the number of days of queen caging is increased.

Considering the estimated adult honey bee populations in the four groups before and after treatments (Table I), the "Apiguard®" group shows a higher survival rate (86.4%). This result was confirmed by other researchers (Gregorc and Planinc 2005; Melathopoulos and Gates 2003) and could be a consequence of the thymol activity in killing *Varroa* and reducing the parasite's harm on bees in late summer. In fact, in the "Control" group the lower honey bee survival rate (79.9%) could be a consequence of the mite's parasitic activity in the untreated colonies (Yang and Cox-

Table I. Total amount of adult bees per group estimated immediately before and after 2 weeks of the thymol treatment.

	Apiguard® group (n=10)	Queen caging group (n=12)	Apiguard® + queen caging group (n=15)	Control group (n=9)
Before treatment	193,000	285,250	314,000	154,250
Two weeks after treatment	166,750	174,250	161,000	123,325
Survival (%)	86.4%	61.1% ***	51.3% ° ***	79.9%

° $P<0.001$ vs K; *** $P<0.001$ vs Ag

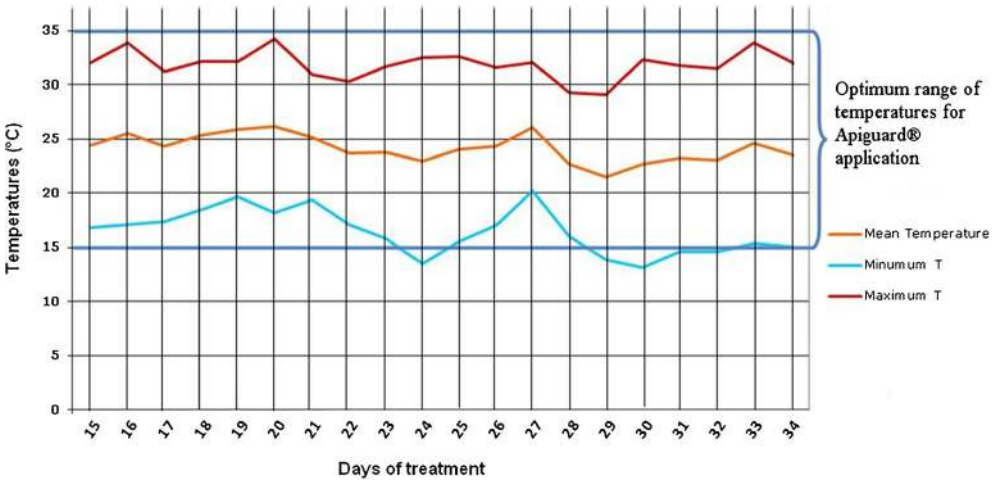


Figure 6. Temperatures recorded in Latina city during the trial (C°). Temperature were detected by the meteorological station of Borgo San Michele (UCEA-Ufficio Centrale di Ecologia Agraria).

Foster 2007). In the "queen caging" group the honey bee survival rate (61.1%) is even worse because of the absence of brood and the subsequent migration of the *Varroa* population from the brood to the adults. Finally, the "Apiguard® plus queen caging" group had the lower adult honey bee survival (51.3%).

Figure 7 shows the performance of the four groups in terms of acaricide efficacy on the x-axis, and survival of adult honey bees on the y-axis. The best performances are obtained when the values are located in the upper-right part of the Cartesian plane: when high acaricide efficacy is accompanied by high honey bee survival. The dispersion of the values obtained from hives of the same group suggests the variability in performance for each treatment. The 21-day queen caging treatment demonstrated high variability both in acaricide efficacy (ranging from 22.9% to 99.3%) and in adult honey bee survival (ranging from 32.4% to 91.3%). However, on its own it is unable to guarantee a satisfactory control against *Varroa* (Table I and Figure 7). The queen caging technique itself presents several drawbacks: it is time consuming because beekeepers spend time identifying and caging the queen. In addition, the queen might be killed either as a result of beekeeper manipulation or due to the lack of re-acceptance by the worker bees when the queen is released into the hive after caging because of a reduction in pheromone. In recent years, this technique has been largely adopted in Italy mainly by small and medium scale beekeepers and, in some cases, by professional beekeepers as well. The "Apiguard®" group showed a considerable variability both in efficacy (ranging from 38.5% to 95.7%) and in adult honey bee survival (ranging from 65.3% to 112.9%), even if the survival percentage resulted higher with respect to "Apiguard® plus queen caging" group (Table I and Figure 7). According to the instructions for use, Apiguard® works best at temperatures above 15°C, but it is also effective at lower temperatures even though it takes longer to evaporate. Indeed Mattila and Otis (2000) triggered with a treatment with Apiguard® during May-June in Ontario (Canada) a 76.2% of acaricide efficacy. Considering the temperatures recorded in our field trial in August (Figure 6), the minimum temperatures were lower than the ideal range of 15°C only in six nights. This happened even though Italy is in Southern Europe, in the Mediterranean area, and even though the trial was carried out in one of the warmest months of the year.

Finally, the "Apiguard® plus queen caging" group had higher acaricide efficacy (96.8%) with less variability (93.1%-98.9%) than the "Apiguard®" and the "Queen caging" groups (Figure 7). This strategy of combining the thymol with queen caging to increase the acaricide efficacy can be considered when thymol is applied in cooler times of the year or in countries where the

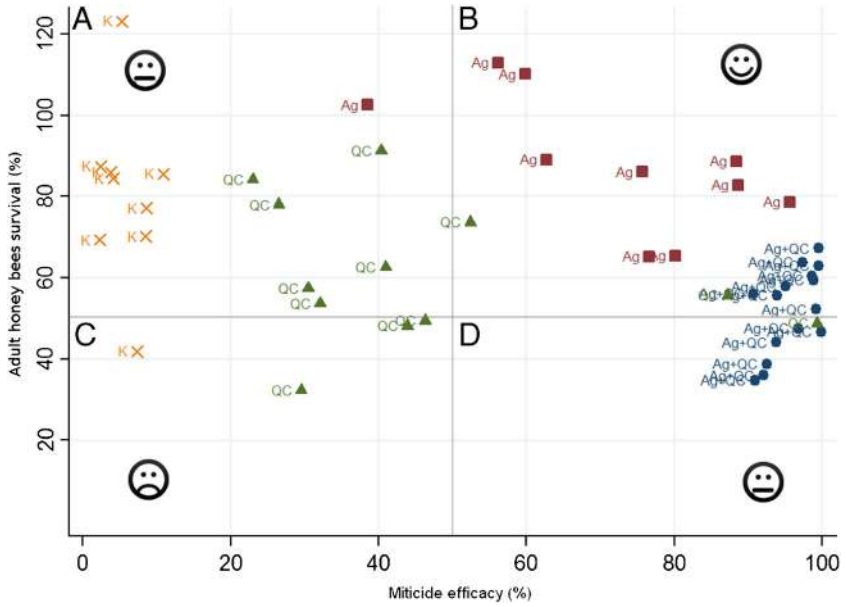


Figure 7. Plot of acaricide efficacy (x-axis) and adult honey bees survival at the end of the treatments (y-axis) in the four groups.

temperatures are lower than in the Mediterranean area (Appendix I), or when the efficacy of the treatment is inadequate (Gregorc 2005). Moreover, the application of thymol (Apiguard®) concurrent with queen caging should be suggested to beekeepers, in cases of high infestation levels of varroosis, but in strong colonies.

In contrast, in case of weak colonies and temperatures >15°C, it should be more useful to suggest to beekeepers the application of thymol only, without caging the queen, especially in late summer, when the reduction in adult honey bee numbers would result in a deficit in the number of winter bees that are essential for the colony to overwinter.

Our procedure did not result in queen mortality in the thymol treated hives. This could also be related to the position of the cage. In fact, in cases of thymol treatments, placing the cage on the lower part of the frame would reduce the mortality of the queens, since they are away from the source of vapours (Pietropaoli and Formato 2015).

In conclusion, the queen caging technique and its acaricide efficacy should be investigated further as a *Varroa destructor* management technique, since it could be adopted by organic beekeepers and it could be able to increase the acaricide efficacy of other organic compounds (oxalic acid, lactic acid, etc.) without using synthetic chemicals.

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OPEN ACCESS

APPENDIX I

Table II. Worldwide distribution of the active ingredient thymol in commercially available products registered for honey bees (font: FAO 2010; EMA/CMDv/497311/2009 2013).

Name of the Commercial Product	Product Active Compounds	Country
AB Var Bio	Thymol	Argentina
Api Life Var	Thymol, Eucalyptus, Camphor, Menthol	Austria, Belgium, Croatia, France, Germany, Hungary, Italy, Libya, Poland, Portugal, Slovakia, Slovenia, Switzerland, United Kingdom, Uruguay, United States of America
Apiguard	Thymol	Albania, Algeria, Australia, Austria, Belgium, Bulgaria, Croatia, Czech Republic, Denmark, Egypt, Estonia, France, Germany, Greece, Hungary, Iran, Iraq, Ireland, Italy, Jamaica, Latvia, Libya, Lithuania, Luxembourg, Macedonia, Mexico, Morocco, Netherlands, New Zealand, Paraguay, Poland, Portugal, Republic of Korea, Romania, Russia, Saudi Arabia, Serbia, Slovakia, Slovenia, Spain, Sweden, Switzerland, Tunisia, Turkey, Ukraine, United Kingdom, United States of America, Uruguay, Uzbekistan
Ecostop lamellae	Menthae, Piperithae Thymolum	Bulgaria
Mehpatika	Thymol	Republic of Korea
Mehpatika Solution	Thymol	Slovakia
Mehpatike	3-p-cimenol, 2,4 hexadien acid, herbs (thyme)	Romania
Natural Var	Thymol	Argentina, Uruguay
Thymol cristalline	Thymol	Switzerland
Thymovar	Thymol	Austria, Belgium, Croatia, Cyprus, Czech Republic, France, Germany, Greece, Hungary, Italy, Netherlands, Poland, Portugal, Republic of Korea, Romania, Slovakia, Slovenia, Spain, Switzerland, United Kingdom

Combinaison du traitement au thymol (Apiguard®) et de la technique d'enfermement de la reine dans la lutte contre *Varroa destructor*

Acari / *Apis mellifera* / lutte contre les acariens / efficacité / produit vétérinaire

Eine Kombination von Thymol-Behandlung (Apiguard®) und Käfigen der Königin zur Bekämpfung von *Varroa destructor*

***Varroa destructor* / Thymol / Apiguard / Königin käfigen / Wirkungsgrad**

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