

Original article

## Effectiveness, persistence, and residue of amitraz plastic strips in the apiary control of *Varroa destructor*

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**Abstract** – An apiary trial was conducted in 1999 in Northern Sardinia (Italy) to evaluate the effectiveness and the persistence of amitraz impregnated in plastic strips against *Varroa destructor* Anderson and Trueman. Twelve colonies of bees derived from *Apis mellifera ligustica* Spin. in Dadant Blatt hives were used; six colonies were treated with 2 strips per hive and the other six were left untreated control. Two methods for the evaluation of treatment efficacy were compared: the percent effectiveness measured as the percent reduction of *V. destructor* infestation in treated hives, and the percent control which took into consideration natural mortality in the control hives. Percent effectiveness was greater than the percent control. Amitraz residues were determined in honey and the plastic strips. No amitraz residue higher than 0.01 mg·kg<sup>-1</sup> was detected in honey. The amitraz content was stable during the trial in plastic strips placed in the colonies, and in the control strips. A higher adult bee mortality in the treated hives was recorded only after the first week of the treatment.

***Varroa destructor* / amitraz / plastic strip / efficacy / persistence / honey / residue / bee mortality**

### 1. INTRODUCTION

In warm areas, due to the almost constant presence of bee brood in the hives, the best chemical control of varroosis is obtained by the use of products with long-term efficacy. Unfortunately, in the last few

years, the development of tolerance to pyrethroids by *Varroa destructor* Anderson and Trueman (Lodesani et al., 1995; Elzen et al., 1998; Floris et al., 2001) has prevented the use of these acaricides. As a consequence, serious loss of colonies has occurred, since the alternative products and

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methods have not been as efficient as pyrethroids.

Recently, the registration in Italy of a new commercial preparation (Apivar<sup>®</sup>, Laboratories Biové SA France, January 1998, Decreto del Ministero della Sanità del 16/12/1998 No. AIC 102481013) made with amitraz (amidine) impregnated in plastic strips has given beekeepers the expectation of having an adequate means to control *V. destructor*. The active ingredient, amitraz, had been previously tested with good results in absence of sealed brood using evaporation or fumigation methods (Frediani and Pinzauti, 1988; Lodesani et al., 1990; Marchetti and Barbattini, 1994).

The aims of this work were (1) to quantify the efficacy of amitraz, as a contact pesticide in plastic strips, against *V. destructor* in beehives, (2) to estimate the persistence of the active ingredient in the plastic strips during the treatment, and (3) to determine some side effects such as bee mortality and residues in honey.

## 2. MATERIALS AND METHODS

### 2.1. Apiary trial

The trial was carried out in an apiary of twenty colonies of bees derived from *Apis mellifera ligustica* Spin., during the autumn (Sept.–Nov.) of 1999 in Northern Sardinia (Sassari). The colonies were maintained in Dandant Blatt hives. In this area, the population of *V. destructor* was shown to be resistant to pyrethroids in a previous study (Floris et al., 2001). Before starting the trial, all colonies were monitored to estimate the consistency of the bee population and the mite infestation level to obtain two homogeneous experimental groups (treated and control) of six hives each.

In the treated group, 2 plastic (composition not given) strips of Apivar<sup>®</sup> (dosage of amitraz, 500 mg per strip) were inserted in the brood chamber of each hive and left for

six weeks. The control group colonies were left untreated.

Before the treatment, the mite infestation levels of worker sealed brood and adult bees were estimated in both treated and control hives, by inspecting 300 cells in a cross-ways manner from three combs total per hive (Floris, 1992) and by brushing about 300 bees from at least three combs total per hive (Ritter and Ruttner, 1980).

During the treatment period, fallen mites and dead adult bees from treated and control hives were counted weekly using a white Vaseline-covered plastic sheet inserted under a screen at the bottom of each hive to count mites, and Gary traps to count dead bees (Gary, 1960). The number of mites that fell onto the bottom of treated hives due to the treatment effect and to the natural mortality was compared with the natural mite mortality of the untreated control hives for an appropriate correction according to the Abbott's formula (Abbott, 1925).

After six weeks, strips were removed from the treated hives and all the sealed brood cells were inspected, both in the treated and control hives, to count the residual mite presence in the brood. For this purpose, the comb were taken from the hives and the visual observations of the sealed cells were made near the apiary to avoid disturbance of the age distribution of the colonies. Finally, all the hives were submitted to two treatments according to the recommendations from the European working group CA3686 (2001): the first one with coumaphos (Perizin<sup>®</sup>) and the second, after a week, with oxalic acid (spray treatment of 50 mL Oxalic acid water solution – 30 g of oxalic acid in 1000 mL distilled water – per hive) (Floris et al., 1998) to count the residual mites in adult bees as above described.

Two parameters were adopted to evaluate the efficacy of the acaricide treatments. The first one was the percent efficacy ( $E\%$ ) calculated as:

$$E\% = 100 [I_t / (I_f + I_t)]$$

where  $I_t$  = the total number of mites collected at the bottom of each treated hive during the treatment period and  $I_f$  = the number of mites collected after the final treatments plus the mites recorded in the sealed brood at the end of the trial. This evaluation was conducted only for treated hives.

The second was the percent control (C%) (Abbott, 1925) calculated as:

$$C\% = (C_s - T_s) / C_s$$

where  $C_s$  and  $T_s$  are the percentages of surviving mites in the control and in the treated hives, respectively; while  $(C_s - T_s)$  is the percentage of mites killed by the treatment. The percentage of surviving mites was calculated as:

$$100 [I_f / (I_f + I_t)]$$

where  $I_f$  and  $I_t$  were already described above.

To evaluate amitraz content of the strips, two disc-shaped portions (ca. 5 mm diam) were taken weekly from each strip using a hole-puncher, for six weeks from the six treated hives. The strip samples were held at  $-20^\circ\text{C}$  until analysis. In the same days, two honey samples (ca. 12 g) were collected from each hive by sucking them with a 50 ml syringe from ca. 100 unsealed cells from three combs with honey in the brood nest close to and far away from the strips.

## 2.2. Chemicals

Analytical amitraz standard was purchased from Ehrenstorfer (Augsburg, Germany). A standard stock solution ( $500\text{ mg}\cdot\text{kg}^{-1}$ ) was prepared in acetone. Triphenyl phosphate (99%) was used as internal standard and was of analytical grade (Janssen, Geel, Belgium). Working standard solutions were obtained by dilution with hexane extracts containing triphenyl phosphate as internal standard at  $0.05\text{ mg}\cdot\text{kg}^{-1}$  from untreated (control) honey. Hexane and acetone were solvents for HPLC (Carlo Erba, Milan, Italy).

## 2.3. Extraction procedure

The following, novel techniques are used:

**Honey** – Ten g of honey were dissolved in 40 g of water, an aliquot of the solution (10 g) was weighed in 40 ml screw-capped tube, 20 ml of hexane were added, and the tube was agitated (30 min) in a rotary shaker. The phases were allowed to separate, and 10 ml of the extract were then evaporated to dryness under nitrogen stream, the residue was dissolved 1 ml of hexane containing internal standard and injected in GC.

**Strips** – Disc-shaped portions of strip were weighed (ca. 60 mg) in a 10 ml screw-capped vial; 10 ml of chloroform were added and the vials were plunged in hot water at  $70^\circ\text{C}$  for 20 min to allow the plastic strip to be dissolved. After cooling at room temperature, the solution was centrifuged, 100  $\mu\text{L}$  of clean liquid was evaporated to dryness under a nitrogen stream, taken up with 10 ml of hexane containing internal standard and injected in a GC.

## 2.4. Apparatus and chromatography

An HRGC TermoQuest Trace 200, with a detector NPD 80 (Termo Quest, Milano, Italy) was used. The column was a fused silica capillary MDN-12 (12% Phenyl-Methyl-Polysiloxane, 30 m  $\times$  0.25 mm id and film 0.25  $\mu\text{m}$ ) (Supelco, Bellefonte, PA, USA). The injector and the detector were at  $230^\circ\text{C}$  and  $300^\circ\text{C}$ , respectively. The sample (2  $\mu\text{L}$ ) was injected in the splitless mode (60 s) and oven temperature was programmed as follows:  $150^\circ\text{C}$  hold 1 min, raised to  $260^\circ\text{C}$  ( $8^\circ\text{C}\cdot\text{min}^{-1}$ ), raised to  $300^\circ\text{C}$  ( $15^\circ\text{C}\cdot\text{min}^{-1}$ ). Helium was the carrier gas and nitrogen was the makeup gas at 1.8 and 20  $\text{mL}\cdot\text{min}^{-1}$ , respectively. The air and hydrogen flows were at 2 and 60  $\text{mL}\cdot\text{min}^{-1}$ , respectively. The operative conditions of the detector were the following: current = 1.72 A; polarisation = 3.5 V. The calibration curves were calculated between peak height and concentration using the

**Table I.** Pre-treatment mite infestation and sealed brood area (mean\*  $\pm$  SD) of experimental hives.

Hive groups	Worker brood infestation (%)	Adult bee infestation (%)	Sealed brood area (cm <sup>2</sup> )
Treated	2.4 $\pm$ 1.2	3.1 $\pm$ 1.6	2444 $\pm$ 357
Control	2.4 $\pm$ 1.2	2.1 $\pm$ 1.6	2610 $\pm$ 401

\* Means in the same column are not significantly different (ANOVA,  $P > 0.05$ ).

external standard method. A good linearity was achieved in the 0.01–0.5 mg·kg<sup>-1</sup> range with a correlation coefficient of 0.9996. Clean-up was unnecessary because there were no interference peaks. The limit of determination (Thier and Zeumer, 1987) was 0.01 mg·kg<sup>-1</sup>.

### 2.5. Recovery assays

Untreated samples of honey were fortified with 0.01, 0.05 and 0.1 mg·kg<sup>-1</sup> of pesticides, and processed according to the procedure described above. At each fortification level, four replicates were analysed. The average recovery was 95% (range 88–110%) with a maximum coefficient of variation (CV) of 11%.

### 2.6. Statistical analysis

Data on fallen mites, adult bee mortality,  $E\%$  and  $C\%$  were subjected to a one-way analysis of variance (ANOVA). The  $E\%$  and  $C\%$  percent values were transformed using the arcsine of the square root to reduce heterogeneity of variances before ANOVA; untransformed means are given in Tables I and II. When  $F$  tests were significant ( $P < 0.05$ ), means were separated by applying the Least Significant Difference (LSD) test ( $P \leq 0.05$ ) (Statgraphics plus, 1998).

## 3. RESULTS

### 3.1. Apiary trial

Data on adult bee and brood infestations, and sealed brood area before treatment are

**Table II.** Efficacy of amitraz treatment against *V. destructor* evaluated with two different methods:  $E\% = 100[I_t / (I_t + I_f)]$  where  $I_t$  = the number of mites collected in each treated hive during the treatment period and  $I_f$  = the number of mites collected after the final treatments;  $C\% = (C_s - T_s) / C_s$  where  $C_s$  and  $T_s$  are the percentages of surviving mites in the control and in the treated hives, respectively.

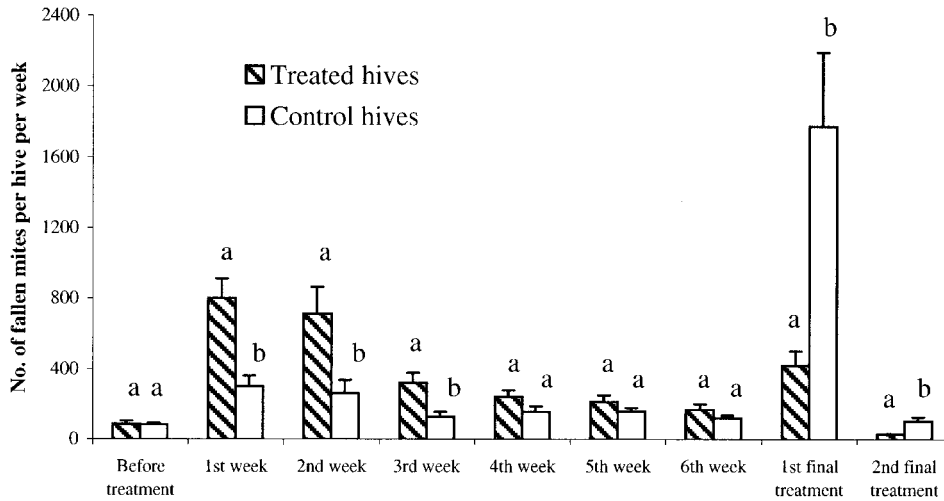
Hives	$E\%$	$C\%$
1	87.3	81.4
2	86.9	80.2
3	80.3	70.2
4	78.8	66.3
5	83.9	74.4
6	85.5	76.9
Means $\pm$ SD	83.8 $\pm$ 3.5	74.9 $\pm$ 5.9

Means are significantly different (ANOVA,  $P < 0.01$ ).

reported in Table I. No significant differences between the two experimental groups ( $P = 0.9813$  for the worker brood;  $P = 0.3066$  for adult bee;  $P = 0.4622$  for the brood area) were found at the beginning of the trial.

The effectiveness evaluated with the two parameters ( $E\%$  and  $C\%$ ) are reported in Table II. The percent effectiveness method produced values significantly higher (83.8  $\pm$  3.5) than the percent control ones (74.9  $\pm$  5.9) ( $F = 1.27$ ;  $df = 2$ ;  $P = 0.03199$ ).

The average trends (means  $\pm$  SE) of fallen mites counted weekly at the bottom of the hives are reported in Figure 1. During the first three weeks after treatment, the number of fallen mites was significantly

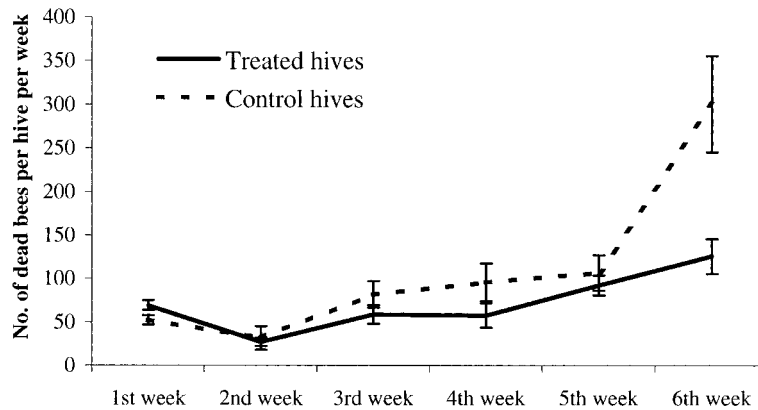


**Figure 1.** Weekly average number  $\pm$  SEM of fallen mites during the treatment period and after final treatments. Within each date, different letters above bars, indicate significant differences among treatments ( $P \leq 0.05$ , LSD test).

higher in the treated hives than in the control ones ( $800 \pm 112$  vs.  $299 \pm 60$ ;  $713 \pm 152$  vs.  $258 \pm 76$ ;  $319 \pm 59$  vs.  $126 \pm 28$ , for the 1st, 2nd and 3rd week, respectively). The two final treatments produced a significantly higher number of fallen mites in the untreated hives than in the treated ones ( $1774 \pm 415$  vs.  $419 \pm 80$  and  $105 \pm 21$  vs.

$28 \pm 3$  fallen mites /hive for the coumaphos and oxalic acid treatments, respectively).

The adult bee mortality during the trial is reported in Figure 2. A statistical difference was observed between treated and control hives by the 1st week ( $F = 4.81$ ;  $df = 1$ ;  $P = 0.0530$ ), when the adult bee mortality



**Figure 2.** Weekly average number  $\pm$  SEM of dead bees during the trial period. Significant differences between treated and control group were observed only at 1st and 6th weeks ( $P \leq 0.05$ , LSD test).

was higher in treated hives probably due to the effect of the treatment. On the contrary, after the 5th week from the treatment a higher bee mortality was recorded in the untreated hives ( $F = 9.89$ ;  $df = 1$ ;  $P = 0.0104$ ) due to the increase of the infestation level as suggested by the moderately strong relationship ( $r = 0.72$ ,  $P = 0.0085$ ) between the infestation level and mortality of adult bees.

### 3.2. Persistence of amitraz in plastic strips

The minimum content of amitraz reported in the label was 500 mg per strip, equal to 3.33% (w/w) considering that each strip weighed 15 g. Data on amitraz in the strips are reported in Table III. Three control strips were kept in the dark in the laboratory. The initial content of amitraz per strip was on average 563 mg, ranging between 485 and 660 mg. This variability could depend on the analytical procedure and on a possible non homogeneous distribution of acaricide in the strip. However, standard deviations

were not high, ranging between 2 and 13%. During the six weeks of experiment, the amitraz residues in the hive and control strips were stable and no significant differences between initial and final content were detected.

### 3.3. Residues in honey

Maximum residue levels (MRLs) of amitraz in honey are fixed nationally. They are generally very low and correspond to the detection limit of the analytical method.

In Italy, Germany and Switzerland, MRLs are 0.01 mg·kg<sup>-1</sup>, in Netherlands 0.02 mg·kg<sup>-1</sup>, while in EU and USA they are 0.20 and 1.00 mg·kg<sup>-1</sup> respectively (Piro, 1999). The analytical procedure used in this work is original and allows for amitraz determination by GC-NPD as the active ingredient at 0.01 mg·kg<sup>-1</sup>, while in the methods commonly used to determine amitraz its total conversion into 2,4 dimethylaniline was necessary (Hornish et al., 1984; Taccheo Barbina et al., 1988; Franchi and Severi, 1989). Other methods used to

**Table III.** Residue of amitraz in strips (mg/strip) during the experiment (controls 1, 2, 3 refer to 3 strips each in each group, kept in the dark in the laboratory).

Hives	Week						
	0	I	II	III	IV	V	VI
1	627	493	504	580	624	527	586
2	522	529	515	571	562	452	557
3	628	533	493	553	602	565	589
4	514	505	538	563	572	558	584
5	485	544	540	592	527	575	569
6	660	564	542	555	590	534	573
Means ± SD	573 ± 74	528 ± 26	522 ± 21	569 ± 15	580 ± 34	535 ± 45	576 ± 12
Control							
1	619	521	541	–	–	–	557
2	515	534	496	–	–	–	596
3	525	511	542	–	–	–	571
Means ± SD	553 ± 57	522 ± 12	526 ± 26	–	–	–	575 ± 20

determine amitraz and its metabolites by HPLC and GC-MS are less sensitive (Bogdanov, 1988; Jiménez et al., 1997). In all honey samples no amitraz residue higher than  $0.01 \text{ mg}\cdot\text{kg}^{-1}$  was detected.

#### 4. DISCUSSION

Under our experimental conditions, the efficacy of amitraz in plastic strips against *V. destructor* in colonies with brood varied with the evaluation method. The higher values obtained with the "percent efficacy" (*E%*) method, in comparison with the "Percent control" (*C%*) one, were probably due to the effect of natural mite mortality which was not taken into account in the *E* parameter.

The presence of a considerable amount of amitraz residue in the plastic strips during the whole period of treatment (6 weeks) indicates both a high persistence of this active ingredient and its low removal by bees. Other pesticides formulated in strips, such as fluralinate and flumethrin (Cabras et al., 1997; Floris et al., 2001) also showed high persistence.

No residue higher than  $0.01 \text{ mg}\cdot\text{kg}^{-1}$  was detected in honey. Even in other studies, in which amitraz as administered by aerosol, very low or no residues were found (Avila et al., 1990; Lodesani et al., 1992; Fernandez et al., 1997). For instance, in East Germany, out of 330 honey samples analysed in 1986–1990, only 8.5% them contained more than  $0.05 \text{ mg}$  of amitraz· $\text{kg}^{-1}$  (Hemmerling et al., 1991). The low residue values, generally determined in honey even when amitraz is directly administered in the hive, are probably due to its fast degradation (2–4 weeks) (Jiménez et al., 1997).

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**Résumé – Efficacité et persistance de l'amitraz dans les bandelettes en plastiques pour lutter au rucher contre *Varroa destructor* et résidus dans le miel.** Un essai en rucher a été fait en 1999 dans le nord de la Sardaigne (Italie) pour évaluer l'efficacité et la persistance de l'amitraz dans les bandelettes en plastique utilisées contre l'acarien *Varroa destructor* Anderson and Trueman. Douze colonies d'*Apis mellifera* L. dans des ruches Dadant-Blatt ont été utilisées : six colonies ont été traitées avec 2 bandelettes par ruche, les six autres ont servi de témoin (non traitées). Avant le traitement les niveaux d'infestation par l'acarien du couvain operculé d'ouvrières et des abeilles adultes avaient été estimés dans les deux groupes de ruches par inspection de 300 cellules réparties sur 3 rayons par ruche de façon croisée et par brossage d'environ 300 abeilles sur au moins trois rayons par ruche. Pendant la période de traitement, on a compté chaque semaine les acariens tombés sur une feuille de plastique enduite de vaseline et glissée sous un grillage sur le fond de ruche, ainsi que les abeilles adultes mortes récupérées dans des trappes Gary. On n'a pas trouvé de différence significative en début d'essai entre les deux groupes expérimentaux, que ce soit pour l'infestation des abeilles adultes ou celle du couvain.

On a comparé deux méthodes pour évaluer l'efficacité du traitement : le pourcentage d'efficacité mesuré en tant que pourcentage de réduction de l'infestation par l'acarien dans les ruches traitées et le pourcentage de contrôle en tenant compte aussi de la mortalité naturelle des acariens dans les ruches témoin. La méthode du pourcentage d'efficacité a donné des valeurs significativement plus élevées ( $83.8 \pm 3.5$ ) que le pourcentage de contrôle ( $74.9 \pm 5.9$ ) (Tab. II, Fig. 1). Le nombre d'acariens tombés compté chaque semaine sur le fond des ruches était significativement plus élevé dans les ruches traitées que dans les ruches témoins (Fig. 1). Une méthode analytique originale a été utilisée pour déterminer par chromatographie

en phase gazeuse avec un détecteur NPD l'amitraz en tant que matière active à  $0,01 \text{ mg}\cdot\text{kg}^{-1}$ , alors que dans les méthodes communément utilisées pour déterminer l'amitraz sa conversion totale en 2,4 diméthylaniline était nécessaire.

On n'a pas trouvé de résidus au-delà de  $0,01 \text{ mg}\cdot\text{kg}^{-1}$  dans aucun des échantillons de miel. Au cours des semaines de traitements les teneurs en amitraz dans la ruche et les bandelettes d'amitraz sont restées stables et n'ont pas montré de différences significative entre la teneur initiale et la teneur finale (Tab. III).

Une mortalité adulte plus élevée dans les ruches traitées n'a été enregistrée qu'après la première semaine de traitement. Au bout de la cinquième semaine de traitement, une mortalité plus élevée a été enregistrée dans les ruches non traitées probablement due à l'augmentation du niveau d'infestation.

#### ***Varroa destructor* / amitraz / efficacité / persistance / miel / résidu**

**Zusammenfassung – Wirksamkeit und Wirkungsdauer von Amitraz in Plastikstreifen bei der Bekämpfung von *Varroa destructor* und seine Rückstände im Honig.** In Nord-sardinien wurde 1999 in einem Bienenstand ein Versuch durchgeführt, um die Wirksamkeit und Wirkungsdauer von Amitraz in Plastikstreifen bei der Bekämpfung von *Varroa destructor* zu bestimmen. Zwölf Völker von *Apis mellifera* wurden in Standard Dadant-Blatt Beuten eingesetzt. Sechs Völker wurden mit 2 Streifen behandelt, die anderen 6 Völker (unbehandelt) dienten als Kontrolle. Vor der Behandlung wurde der Befallsgrad von Arbeiterinnen und Brut sowohl bei den Versuchsvölkern als auch bei den Kontrollen bestimmt, indem man jeweils insgesamt 300 Zellen von 3 Brutwaben in sich kreuzenden Reihen inspizierte und etwa 300 Bienen von mindestens 3 Waben pro Volk abkehrte. Während der Behandlung wurden die abgefallenen Milben und toten Bienen

wöchentlich gezählt. Die Milben wurden auf einer mit Vaseline bestrichenen weißen Plastikfolie unter einem Netz am Boden von jedem Volk gezählt, die Bienen mit Gary Fallen. Am Beginn des Versuchs gab es keine signifikanten Unterschiede im Befall von Brut und Bienen bei den beiden Versuchsgruppen (Tab. I).

Zwei Methoden zur Bewertung der Wirksamkeit der Behandlung wurden verglichen: die prozentuale Wirksamkeit wurde als Prozent der Abnahme der *Varroa* Infektion in den behandelten Völkern gemessen sowie als Prozent gegenüber der Kontrolle, um den Effekt der natürlichen Milbensterblichkeit zu berücksichtigen. Mit der Methode der prozentualen Wirksamkeit erhielten wir signifikant höhere Werte ( $83,8 \pm 3,5$ ) als bei den Prozenten gegenüber der Kontrolle ( $74,9 \pm 5,9$ ) (Tab. II, Abb. 1). Die Anzahl der abgefallenen Milben, die wir wöchentlich auf den Bodeneinlagen der Völker zählten, war signifikant höher in den behandelten Völkern als den Kontrollen (Abb. 1).

In dieser Arbeit wurde eine neue analytische Methode für die Bestimmung von Amitraz mit dem GC benutzt unter Verwendung von NPD als aktiver Substanz mit einer Empfindlichkeit bis  $0,01 \text{ mg}\cdot\text{kg}^{-1}$ ; bei den normalerweise benutzten Methoden musste Amitraz vollständig in 2,4 Dimethylanilin umgewandelt werden. In keiner Honigprobe wurden mehr als  $0,01 \text{ mg}\cdot\text{kg}^{-1}$  Amitrazrückstände nachgewiesen. Während des sechswöchigen Versuchs blieb der Amitrazgehalt im Stock und bei den Kontrollplastikstreifen unverändert und es gab keine signifikanten Unterschiede zwischen dem Ursprungs- und Endgehalt (Tab. III). Nur in der ersten Woche der Behandlung wurde eine erhöhte Bienensterblichkeit festgestellt. In der 5. Woche nach Beginn der Behandlung wurde eine höhere Bienensterblichkeit bei den Kontrollvölkern festgestellt, vermutlich auf Grund der Erhöhung des Milbenbefalls.

#### ***Varroa destructor* / Amitraz / Plastikstreifen / Wirksamkeit / Persistenz / Honig / Rückstände / Bienensterblichkeit**



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