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Original article

The varroacidal action of propolis: a laboratory assay

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Abstract – The action of propolis (bee glue) against the ectoparasitic mite *Varroa destructor* Anderson and Trueman has been investigated and showed narcotic and lethal effects. Length of narcosis and rate of mortality depended on the extraction procedure, concentration of propolis and contact time. Propolis extracted with 70% ethanol was found to be highly toxic, a 10% (w/v) propolis resulting in 100% mortality with a brief contact time of 5 s. In addition, the effect of propolis on the metabolic rate of the mites has been investigated calorimetrically. Even sublethal propolis concentrations without varroacidal effects and of only short lasting narcotic effects resulted in a significant reduction in the heat production rate, indicating weakening of the mites.

***Varroa destructor* / varroatosis / varroacides / propolis / *Apis mellifera* / calorimetry**

1. INTRODUCTION

The threat of honeybee infestation by *Varroa destructor* Anderson and Trueman forces beekeepers in many parts of the world to treat their colonies with acaricides, which are associated with drawbacks. The most serious drawbacks are the build up of residues in bee products (Kubik et al., 1995; Wallner, 1995; Stürz and Wallner, 1997; Bogdanov et al., 1998; Wallner, 1999) and the development of resistant mite strains. *V. destructor* strains have been reported to be resistant to fluvalinate and flumethrin (Colombo et al., 1993; Eischen, 1995; Lodesani et al., 1995; Milani, 1995; Baxter et al., 1998), coumaphos (Milani and Della Vedova, 1996; Spreafico et al., 2001), bromopropylate and chlordimeform (Ritter and Roth 1988), and to amitraz (Elzen et al., 2000). The problems associated with the use of acaricides provide considerable incentive to develop new treatment strategies and screening for potential acaricides that

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minimize these problems. Natural products having components with various modes of action might provide effective solution to the problem of varroatosis (Mutinelli et al., 1997; Imdorf et al., 1999). One of such natural products is propolis (bee glue), a complex mixture of several compounds collected by honeybees from plants, mixed with wax and used in the construction and protection of the beehive (Ghisalberti, 1979).

Literature on the acaricidal or insecticidal action of propolis is very limited. It has been assumed that components of nectar, pollen and propolis may adversely affect the development of *V. destructor* in the hive of some bee populations (Amrin et al., 1996, http: //www.wvu.edu/~agexten/varroa/varroa2.htm#Comments). Some authors proposed that some flavonoid components of propolis have insecticidal or at least insectstatic (inhibition of insect larval development) effects (König and Dustmann, 1988). Even though the anaesthetic and lethal action of propolis against *V. destructor* has been briefly mentioned in the literature (Schkurat and Poprawko, 1980), its potential acaricidal use, as far as our knowledge is concerned, is not well investigated.

2. MATERIALS AND METHODS

2.1. Propolis extraction and preparation

Propolis samples used in our experiments were obtained from scrapings of beehives in the garden of the Institute of Zoology, Free University of Berlin, Germany. Pre-weighed and frozen samples were homogenised using a coffee mill (type MZ Moulinex, France). The homogenate powder was then extracted in 70% or 40% ethanol. The extraction in 40% ethanol was intended to procure components to be used in less concentrated ethanol solution to minimize the effect of ethanol on the experimental organisms. For effective extraction, the propolis powder was suspended in the corresponding ethanol solution in a ratio of 1:9 (w/v) (Strehl et al., 1994). The suspension was extracted in a rotary evaporator (Rotationsverdampfer W-micro, Heidolf, Mannheim, Germany) at 60 ^{o}C for 2 h. The suspension was then cooled at room temperature for ca. 1 h and suction filtered. The filtrate was dried in an incubator at 40 ^{o}C to weight constancy, which was achieved in two weeks time. The yield of extraction was 58% (w/w) for the extraction in 70% ethanol and 19% (w/w) in 40% ethanol.

The 70% ethanol extract was used in 55% ethanol (solution B hereafter) in the bioassay to reduce the effect of strong ethanol solution on the experimental organisms. The little amount of precipitation observed while suspending solution B was brought into solution by agitation. The 40% ethanol extract was used in the same ethanol concentration (solution A hereafter) for the bioassay. The concentrations used in the bioassay were 20%, 15%, 10%, 7.5%, 5% (w/v) solution A and 10%, 7.5%, 5%, 2%, 1%, 0.5% (w/v) solution B.

Acaricide residue analysis of the propolis sample used in our experiments was done at the Landesanstalt für Bienenkunde der Universität Hohenheim, Stuttgart, Germany.

2.2. Mite collection

Mites were collected from infested colonies in the garden of the Institute of Zoology, Free University of Berlin, Germany. The colonies were treated only at the beginning of autumn of the preceding year with formic acid. The experiments were conducted in summer 2000. Adult *V. destructor* females were collected from capped healthy drone brood cells by opening and inspecting individual cells. During the collection process, mites were kept in a Petri dish on bee larvae or pupae to avoid starvation. Newly moulted adult mites, identified

by their pale colour, relatively smaller size, and weak locomotion were excluded from the experiment since they may have had a different response as hardening of the cuticle was still in progress. Mites which seemed weak and abnormal were discarded.

2.3. Bioassay

Treatment of the mites was done by applying 400 µL of a given concentration of propolis on a 3 cm × 3 cm tissue paper (Kimwipes® Lite 200, Kimberly – Clark®) in a Petri dish and by immediately placing six mites per experiment on the wetted tissue paper. To observe the effect of contact time of propolis on the activity of *V. destructor,* the following treatment times were used: 5, 10, 20, 30, 40, 60, 75, 90 s for the treatment with solution B and 20, 40, 60, 90 s for the treatment with solution A. The treatment was stopped after the allocated time by removing the mites with the tissue paper from the Petri dish and immediately placing them on a pad of paper towel for 1 min to blot the excess fluid from the surfaces. They were then transferred to a clean Petri dish and their activity observed under a dissecting lens every five minutes for the first hour, every 10 minutes for the next one hour and every 30 minutes for the next two hours. No activity change was observed after an incubation period of two hours. All treatments were done at 30 °C and the treated mites were incubated at 34 ± 0.5 °C. Control experiments for each experimental group were done by treating the mites for the corresponding time with 40% or 55% ethanol solution and also distilled water.

An individual was considered inactivated if it showed no leg movement or movement of any body part when gently prodded with a probe. If it showed movement it was counted as alive, irrespective of whether it was partially paralysed or normal. If the inactivation lasted more than four hours after the treatment time the mites were considered dead, since further incubation did not show any activity change. Each treatment was repeated five times and the mean values, and in some cases the mean ± S.D. values, were used in the presentation of results.

2.4. Calorimetric experiments

The bioassay method enabled us to assess the action of propolis by counting the number of active/inactivated (dead) individuals but it did not allow us to evaluate the extent of the effect on the surviving and weakened individuals. Thus, we conducted calorimetric experiments to observe to what extent a certain sublethal propolis dose affects the metabolic rate of the mites. The calorimeter used was a Biocalorimeter, B.C.P-600, MV Messgeräte Vertrieb, München, Germany, of sensitivity 50 µV/mW and a vessel volume of 20 cm^3.

To compare the heat production rate before and after treatment, 20 to 25 untreated mites per experiment were put in the calorimetric vessel and their heat production rate was recorded for 4 hours. Recording was then stopped, and the mites were removed from the calorimeter and were treated with propolis. The treatment was done for 30 s with solution B and for 60 s with solution A. The treated mites were put back in the calorimetric vessel and their heat production rate was recorded for 8 to 11 hours. Each experiment was repeated five times and the mean ± S.D. values were used in the presentation of results.

3. RESULTS

The residue analysis of propolis, with a detection limit of 1 mg/kg, showed that the propolis sample was free of any acaricide contaminant.

The controls of both solution A and solution B showed narcotic effects for a short

Table I. Effect of contact time on the activity of *Varroa destructor* under treatment with different concentrations of solution B (a) and solution A (b). Six mites per experiment, n = 5, percentages of the mean values are presented here.

a.

Concentration of propolis (% w/v)	Contact time (s)	Percentage of inactivated mites at each observation time (min)			
		0	30	60	120
Control	10	53.3	0.0	0.0	0.0
	30	80.0	0.0	0.0	0.0
	60	100	0.0	0.0	0.0
0.5	10	100	0.0	0.0	0.0
	30	100	6.7	3.3	3.3
	60	100	3.3	3.3	3.3
2.0	10	100	6.7	6.7	6.7
	30	100	10.0	10.0	10.0
	60	100	16.7	6.7	6.7
5.0	10	100	53.3	50.0	36.7
	30	100	70.0	56,7	50.1
	60	100	83.3	66.7	63.7
7.5	10	100	53.3	50.0	43.3
	30	100	76.7	63.3	53.3
	60	100	83.3	76.7	73.3

b.

Concentration of propolis (% w/v)	Contact time (s)	Percentage of inactivated mites at each observation time (min)			
		0	30	60	120
Control	20	46.7	0.0	0.0	0.0
	40	49.1	0.0	0.0	0.0
	60	50.0	0.0	0.0	0.0
5	20	45.7	0.0	0.0	0.0
	40	46.7	0.0	0.0	0.0
	60	53.3	0.0	0.0	0.0
10	20	48.0	10.0	8.3	8.3
	40	53.3	10.0	10.0	10.0
	60	60.0	23.3	16.7	16.7
15	20	50.0	16.7	13.3	13.3
	40	52.0	23.3	16.7	13.3
	60	56.7	30.0	26.7	23.3
20	20	55.0	28.5	21.0	21.0
	40	56.7	26.7	25.0	23.3
	60	65.0	33.3	33.3	33.3

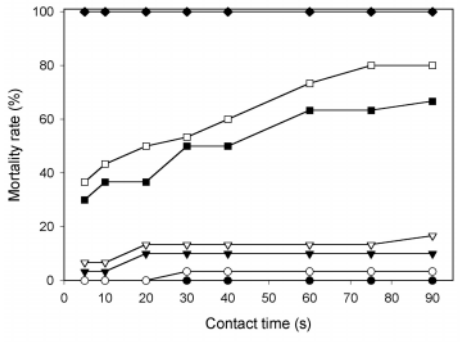

Figure 1. Influence of contact time on the mortality rate of *Varroa destructor* under treatment with different concentrations (% w/v) of solution B. Six mites per experiment, n = 5, percentages of the mean values are presented here. ●- control, ○- 0.5%, ▼- 1%, ▽- 2%, ■- 5%, □- 7.5%, ◆- 10% propolis. 4 h after treatment.

period after treatment. In both cases, this effect lasted for less than five minutes; i.e., 100% of the mites recovered with in the mentioned time interval. The proportion of narcotised mites just after the treatment (zero observation time) ranged from 46.7 ± 7.5% to 53.3 ± 11.8% for control of solution A at 20 s and 90 s contact times, respectively. In the case of control of solution B, 53.3 ± 13.9% to 100% of the mites were narcotised at treatment times of ≤ 10 s and ≥ 60 s, respectively. Even though the control mites of solution A were narcotised shortly after treatment, their metabolic activity was not significantly affected after recovery ($P > 0.05$), being 13.1 ± 1.1 (μW/mg) and 13.0 ± 1.4 (μW/mg) before and after treatment, respectively. The control mites of solution B, however, showed a significant effect ($P < 0.05$) on the metabolic activity resulting in a 13% drop of the specific heat production rate from 13.6 ± 1.8 (μW/mg) to 11.8 ± 2.2 (μW/mg) after treatment (Fig. 3a, b). Contact with water had no effect at all.

Treatment of *V. destructor* with solution B showed that 100% narcosis was achieved for some minutes immediately after treatment, regardless of concentration and contact time. With lower concentrations of solution B (0.5%, 2%) narcosis lasted only briefly (Tab. Ia) and most mites recovered from narcosis in the first 30 minutes after treatment. Narcosis with higher concentrations of solution B (5%, 7.5%) lasted longer and some mites recovered after 60 minutes (Tab. Ia). Treatment of *V. destructor* with solution A resulted in an initial narcosis of 45% to 65% (Tab. Ib), which was lower than the 100% narcosis of solution B.

Even though some mites recovered from narcosis, others could not recover at all. Mites that did not recover in the first 4 h after treatment were considered dead. The effect of different propolis concentrations and contact time on the mortality rate of *V. destructor* is shown in Figure 1 and Figure 2. The varroacidal action of propolis increases with increasing concentration and contact time except for lower concentrations of solution B. Treatment of mites with 10% (w/v) solution B resulted in 100% mortality regardless of the treatment time, indicating its high toxicity with the

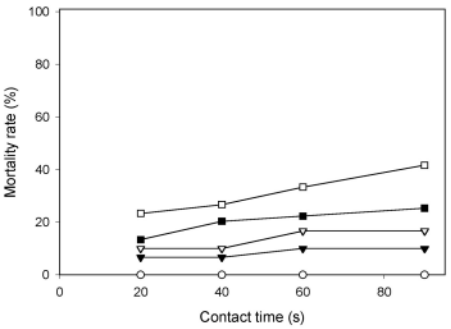

Figure 2. Influence of contact time on the mortality rate of *Varroa destructor* under treatment with different concentrations (% w/v) of solution A. Six mites per experiment, n = 5, percentages of the mean values are presented here. ●- control, ○- 5%, ▼- 7.5%, ▽-10%, ■- 15%, □- 20%. The control experiment and 5% had no lethal effects, values overlapping at the x-axis. 4 h after treatment.

slightest contact (Fig. 1). Treatment of mites with solution A, even with a concentration of 20% (w/v) resulted in a mortality rate of less than 50% (Fig. 2).

Comparison of the specific heat production rates before and after treatment with both solutions showed that even those concentrations that did not have a considerable effect on the mortality rate affected the heat production rate significantly ($P < 0.05$, to $P < 0.001$, Fig. 3a, b).

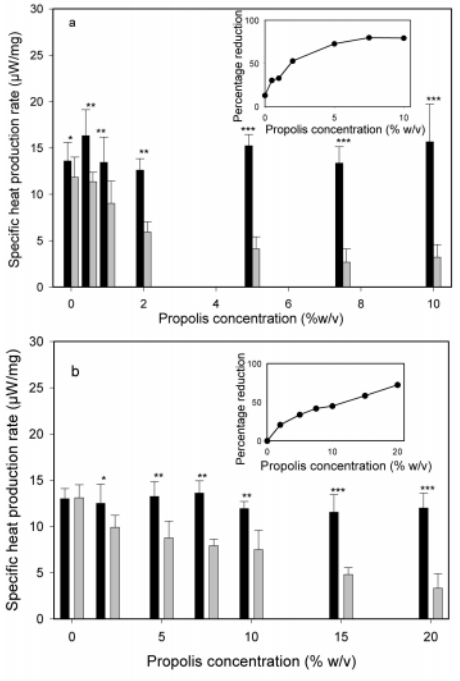

Figure 3. Effect of different concentrations (% w/v) of solution B (a) and solution A (b) on the mean specific heat production rate (mean ± S.D.) of *Varroa destructor* mites. 20 to 25 mites per experiment, n = 5. The inset in each graph, extracted from the corresponding graph, is a curve of the percentage reduction in the mean specific heat production rate versus propolis concentration of treatment. ■- before treatment, □- after treatment. The values at zero concentration are treatments with the corresponding ethanol solutions (controls). Significance levels of $P < 0.05$ –*, $P < 0.01$ –**, $P < 0.001$ –*** (Paired sample t-test).

4. DISCUSSION

Treatment of mites with propolis causes narcosis and death. The narcotic effect of propolis on different animals has already been mentioned in the literature (Prokopovich et al., 1956; Prokopovich, 1957). As seen from our experimental results, treatment of mites with solution B was more effective than the corresponding treatment with solution A. The most plausible explanation for these differences is that solution B was extracted in 70% ethanol whereas solution A was extracted in 40% ethanol.

Even though the control experiments of solution A and solution B showed differences in the percentage of narcotised mites shortly after treatment and in the heat production rates, there was no observed mite mortality in both cases. Unlike the controls, the treatments with the two propolis solutions showed considerable differences in their effect on narcosis and heat production rate and on the mortality rate of the mites. These results indicate that the observed difference in mite mortality between the two treatments was not due to the concentration of the alcohol, but rather to the difference in the ingredients of the two solutions.

The extraction of propolis in 70% ethanol enables one to obtain most of the biologically active hydrophobic components, which could not be extracted in 40% ethanol. This means that solution B was qualitatively and/or quantitatively superior to solution A. Even though the treatment of mites with solution A had only a weak varroacidal effect (Fig. 2), it affected the heat production rate strongly (Fig. 3b); 33.3% mortality rate versus 75.0% reduction in the heat production rate for the treatment with 20% propolis at 60 s contact time.

It has been postulated (König and Dustmann, 1988) that bees must obtain some benefit from the use of propolis in the

beehive, otherwise they would not waste time and energy in collecting it. Our experimental results showed that *V. destructor* mites are highly sensitive to propolis. The varroacidal action of propolis seems to be paradoxical, since propolis and *V. destructor* mites are normally found in the beehive, and the mites walk on thin propolis layers throughout the hive. The most probable explanation for why propolis does not kill the mites in the beehive is that propolis is insoluble in the beehive's interior, due to the fact that most of the components of propolis are water insoluble. The water soluble components of propolis comprise about 2.5%–6.5% of the total, based on the origin of propolis (Neunaber, 1995). As seen in the case of efficacy (both narcosis and mortality) of solution A, in which most of the water soluble and some water insoluble components were extracted, a higher concentration of propolis was needed to observe the varroacidal effect. This shows that even if some of the components of propolis are solubilized in the high humidity in the hive's interior, their concentration is too weak to kill the mites.

If propolis is to be recommended as a varroacidal agent, its use could minimize the contamination of hive products by reducing the use of synthetic acaricides. Except for those allergic to propolis, its presence in hive products may not be considered as a serious contaminant since propolis is used as an additive of cosmetics and in medicine in some countries.

To reduce the amount of inactive components of propolis in hive products, the active varroacidal components of propolis may be isolated and used alone. In addition, it may be worth investigating the synergistic action of propolis with essential oils already being used as varroacides. If propolis is effective in field experiments, and if it has no negative effect on the bees themselves, it may minimize the cost of beekeeping.

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Résumé – Action varroacide de la propolis: test de laboratoire. L'acarien *Varroa destructor* Anderson & Trueman, parasite qui détruit les colonies de l'abeille domestique *Apis mellifera* L., représente donc comme jamais auparavant une menace sérieuse pour l'industrie apicole. Sans traitement médicamenteux la mortalité des colonies peut atteindre 100 %. Actuellement les apiculteurs disposent de différentes méthodes qui comportent de nombreux inconvénients – comme la contamination des produits du rucher ou la sélection de souches d'acariens résistantes. Ces problèmes rendent la recherche de nouveaux produits potentiellement varroacides nécessaire et souhaitable.
Les produits de la nature, qui sont constitués d'une multitude de substances à actions très variées, sont dans ce contexte des candidats pleins de promesses, puisque des résistances à de tels produits sont improbables ou se développeraient plus lentement. La propolis récoltée par les abeilles est un de ces produits. Pour en étudier l'action varroacide, des échantillons de propolis ont été prélevés dans les colonies d'abeilles de l'Institut de Zoologie de l'Université Libre de Berlin et extraits dans de l'éthanol à 40 % et 70 %. L'extrait de propolis issu de l'éthanol à 70 % a été utilisé dans de l'éthanol à 55 % (solution B)

pour un test biologique et l'extrait issu de l'éthanol à 40 % dilué dans de l'éthanol à 40 % (solution A). L'action des deux solutions sur les acariens *V. destructor* a été étudiée à l'aide d'un biotest. Les acariens ont été placés sur des morceaux de papier filtre de 3 cm × 3 cm dans des boites de Petri et arrosés de 400 µl de solution. L'activité des acariens a été suivie durant quatre heures après le traitement. Le nombre d'acariens actifs et d'acariens morts a été pris comme mesure de l'activité de l'extrait de propolis. Puisque cette méthode peut simplement mesurer l'action létale de l'extrait, mais pas l'action sublétale sur le métabolisme des acariens, des études calorimétriques ont été entreprises pour mesurer le taux métabolique.

Le traitement des acariens *V. destructor* à la propolis a provoqué leur narcose et finalement leur mort. L'action narcotique et varroacide de la solution B a été plus forte que celle de la solution A. Une dilution à 10 % (poids/volume) de la solution B a provoqué 100 % de mortalité dès le temps de contact le plus court (5 s). En comparaison l'action d'une dilution à 20 % (poids/volume) de la solution A même après une durée de contact de 90 s était beaucoup plus restreinte avec un taux de mortalité de 42 %. Ce résultat indique que la propolis extraite à l'éthanol à 70 % est quantitativement et/ou qualitativement la plus efficace. Avec une dilution à 20 % de la solution A et une durée de contact réduite à 20 s, seuls 23 % des acariens meurent. Une dilution à 5 % de la solution A avec une durée de contact de 90 s n'a aucune action létale. Ces résultats montrent que l'action de la propolis dépend aussi bien de la durée de contact que de la concentration. Les concentrations de propolis qui n'étaient que faiblement varroacides avaient une nette action sur le taux métabolique. Une dilution à 5 % (poids/volume) de la solution A, sans action létale, réduisait néanmoins le taux métabolique de 34 %. Ce résultat indique que des dosages sublétaux de propolis exercent une action préjudiciable pour les acariens *V. destructor.*

***Varroa destructor* / varroacide / propolis / calorimétrie**

Zusammenfassung – Die varroazide Wirkung von Propolis: eine Laboruntersuchung. Die Milbe *Varroa destructor* Anderson und Trueman parasitiert und zerstört Völker der westlichen Honigbienen *Apis mellifera* L. und stellt daher nach wie vor eine ernsthafte Bedrohung der Bienenindustrie dar. Ohne medikamentöse Behandlung kann die Koloniesterblichkeit bis zu 100% betragen. Zur Zeit stehen den Imkern mehrere Behandlungsmethoden zur Verfügung, von denen viele Nachteile – wie die Kontamination von Bienenprodukten und die Selektion von resistenten Varroastämmen – mit sich bringen. Die hier genannten Probleme machen die Suche nach neuen potentiellen Varroaziden notwendig und wünschenswert.

Naturprodukte, die aus einer Vielzahl von Substanzen mit unterschiedlicher Wirkweise bestehen sind hierbei aussichtsreiche Kandidaten, da Resistenzen gegen solche Produkte unwahrscheinlich sind oder langsamer entwickelt werden können. Das von den Honigbienen gesammelte Kittharz (Propolis) stellt eine solche natürliche Substanz dar. Um die varroazide Wirkung von Propolis zu untersuchen, wurden Propolisproben von Bienenvölkern am Institut für Zoologie der Freien Universität Berlin genommen und in 40 % und 70 % Ethanol extrahiert. Der Propolisextrakt aus der 70 % Ethanollösung wurde in einer 55 % Ethanollösung im Biotest appliziert (Lösung B), und der Propolisextrakt aus der 40 % Lösung in einer 40 % Ethanolverdünnung (Lösung A).

Beide Lösungen wurden in einem Biotest in ihrer Wirkung auf Milben untersucht. Die Varroamilben wurden auf ein 3 cm × 3 cm großes Filterpapier, das sich in einer Petrischale befand, aufgebracht und mit 400 µl

Propolislösung benetzt. Nach der Behandlung wurde die Aktivität der Milben 4 h lang protokolliert. Als Maß für die Wirksamkeit der Propolisextrakte wurde die Zahl der inaktiven und toten Milben aufgenommen. Da diese Methode lediglich die letale Wirkung der Extrakte, nicht aber die subletale Wirkung auf den Stoffwechsel der Milben erfassen konnte, wurden zusätzlich kalorimetrische Versuche zur Messung der Stoffwechselrate der Milben unternommen.

Die Behandlung von Varroamilben mit Propolis führte zu Narkose und schließlich dem Tod der Milben. Die narkotische Wirkung und die Mortalität der Milben waren abhängig von der Extraktionsmethode, der Propoliskonzentration und der Kontaktzeit.

Lösung B hatte im Vergleich zu Lösung A eine stärkere narkotische und varroazide Wirkung. Eine 10 % (w/v) Verdünnung von Lösung B rief bei den Milben bereits nach der kürzesten Kontaktzeit von 5 s eine 100 % Mortalität hervor. Verglichen hiermit war die Wirkung einer 20 % (w/v) Verdünnung von Lösung A selbst nach einer langen Kontaktzeit von 90 s mit einer resultierenden Mortalitätsrate von 42 % deutlich geringer. Dieses Ergebnis deutet darauf hin, dass in 70 % Ethanol extrahiertes Propolis quantitativ und/oder qualitativ am wirkungsvollsten ist. Bei einer Kontaktzeit von 90 s verursachte eine 20 % (w/v) Verdünnung von Lösung A eine Mortalitätsrate von 42 %, bei einer geringeren Kontaktzeit von 20 s starben lediglich 23 % der Milben. Eine 5 % (w/v) Verdünnung von Lösung A hatte bei einer Kontaktzeit von 90 s keine letale Wirkung. Diese Ergebnisse zeigen die Abhängigkeit der Propoliswirkung sowohl von der Kontaktzeit als auch der Konzentration. Auch Propoliskonzentrationen, die nur schwach varroazid waren, hatten einen deutlichen Einfluss auf die Stoffwechselrate. Eine 5 % (w/v) Verdünnung von Lösung A hatte keine letale Wirkung auf die Milben, verursachte aber eine Reduktion der Stoffwechselrate um 34 %. Dieses Ergebnis weist darauf hin, dass auch subletale Propolisdosierungen eine nachteilige Wirkung auf Varroamilben haben.

***Varroa destructor* / Varroatose / Varroazide / Propolis / Kalorimetrie**

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