3.0 The Antivarroa Action of Propolis: a Laboratory Assay

3.1 Abstract

Effect of the ethanol extract of propolis (bee glue) against the ectoparasitic mite *Varroa destructor* (Anderson and Trueman) has been investigated and showed narcotic and lethal actions. 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 Varroa mites has been investigated calorimetrically. Even sublethal propolis concentrations without varroacidal effects, and of only short lasting narcotic effects, resulted in a significant reduction of the specific heat production rate, indicating that the mites are weakened.

3.2 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, however, 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 emergence of resistant mite strains. Varroa strains resistant to the different types of acaricides in use today have been reported from different parts of the world. Resistance of mites to fluvalinate and flumethrin have been reported in Europe and the United States (Colombo et al. 1993, Eichen 1995, Lodesani et al. 1995, Milani 1995, Baxter et al. 1998), to coumaphos in Italy and Switzerland (Milani and Della Vedova 1996), to amitraz in the United States (Elzen et al. 2000), and to bromopropylate and chlordimeform in Europe (Ritter and Roth 1988).

The above mentioned problems associated with the use of acaricides provide considerable incentives to develop new treatment strategies and screening for potential acaricides that minimize these problems. Natural products having different components with various modes of action might provide effective solutions to the problems of varroosis (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 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 rather than emergence of natural resistance (Amrin et al. 1996, http://www.wvu.edu/agexten/varroa.htm). It has been suggested that some flavonoid components of propolis have insecticidal or at least insectistatic (inhibition of insect larval development) effects (König and Dustmann 1988). Even though the anaesthetic and lethal actions of propolis against *V. destructor* have been briefly mentioned in the literature (Schkurat and Poprawko 1980), its potential acaricidal use is not yet investigated.

3.3 Materials and Methods

3.3.1 Propolis extraction and preparation

Propolis samples used in the present experiments were obtained by scrapping off the frames from 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 °C for 2 h. The suspension was then cooled at room temperature for ca. 1 h and then suction filtered. The filtrate was dried in an incubator at 40 °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 in order 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 5%, 7.5%, 10%, 15%, 20% (w/v) solution A and 0.5%, 1%, 2%, 5%, 7.5%, 10% (w/v) solution B.

As the presence of acaricide residues in the propolis sample, due to previous treatments of the colony, may introduce artefacts, acaricide residue analysis was carried out to assure the propolis quality. The residue analysis of the propolis sample was done at the Landesanstalt für Bienenkunde der Universität Hohenheim, Stuttgart, Germany.

3.3.2 Mite collection

Mites were collected from infested colonies, treated only at the beginning of autumn of the preceding year with formic acid, in the garden of the Institute of Zoology, Free University of Berlin, Germany. The experiments were conducted in summer 2000. Adult Varroa females were collected from capped healthy drone brood by opening and inspecting individual cells. During the collection process mites were kept in a Petridish on bee larvae or pupae in order 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 a different response as hardening of the cuticle is still in progress. Mites which seemed weak and abnormal were discarded.

3.3.3 Bioassay

Treatment of the mites was achieved by applying 250 µl of a given concentration of propolis on a 3 cm x 3 cm tissue paper (Kimwipes™ Lite 200, Kimberly – Clark™) in a Petridish and by immediately placing six mites per experiment on the wetted tissue paper. In order 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 Petridish, and immediately placing them on a pad of paper towel for 1 min to blot the excess fluid on the surfaces. They were then transferred to a clean Petridish, and their activity was observed under a dissecting lens. Their activity was checked every five minutes for the first hour, every 10 minutes for the next one hour, and every 30 minutes for the next two hours. All treatments were carried out at room temperature (25 °C) and the treated mites were incubated at 35 ± 0.5 °C. Control experiments for each experimental group were conducted by treating the mites for the corresponding time with 40% or 55% ethanol solution and also distilled water.

An individual mite 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, irrespectively of whether it was partially paralysed or normal. If the

inactivity lasted more than four hours after the treatment time the mites were considered dead; further incubation did not show any activity change. Each treatment was repeated five times and the mean ± S.D. values were used in the presentation of results.

3.3.4 Calorimetric experiments

The bioassay method mentioned above enables us to assess the action of propolis only by counting the number of active or inactivated (dead) individuals, but not the extent of the effect on the surviving and weakened individuals. For this reason calorimetric experiments were conducted 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) with a sensitivity of 44.73 $\mu V\ mW^{-1}$ and a vessel volume of 12 cm^3.

In order 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 2 h. Recording was then stopped, and the mites were removed from the calorimeter and treated with propolis. The treatment lasted 30 s with solution B and 60 s with solution A. The treated mites were put back into the calorimetric vessel and their heat production rate was recorded for 4 to 5 h. Each experiment was repeated 5 times and the mean ± S.D. values were used in the presentation of results.

3.4 Results

The acaricide residue analysis of propolis, with a detection limit of 1 $mg\ kg^{-1}$, showed that the propolis sample was free of any acaricide contaminant.

The control treatments of both solution A and solution B, i.e. with only 40% and 55% ethanol, respectively, have shown narcotic effects for a short period after treatment. In both cases this effect lasted < 5min i.e., 100% of the mites recovered within this 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 case of the 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 with the rest lying between these values. Even though the control mites of solution A were narcotised shortly after treatment, their metabolic activity was not significantly affected after recovery ($P > 0.05$); being13.1 ± 1.1 ($\mu W\ mg^{-1}$) and 13.0 ± 1.4 ($\mu W\ mg^{-1}$) before and after treatment, respectively

(Fig. 3.5 a). The control treatments of solution B, however, displayed a significant effect ($P < 0.05$) on the metabolic activity of the mites resulting in a drop of the specific heat production rate from 13.6 ±1.8 (µW mg^{-1}) to 11.8 ± 2.2 (µW mg^{-1}) by 13% after treatment (Fig. 3.5 b). Contact with water had no effect at all.

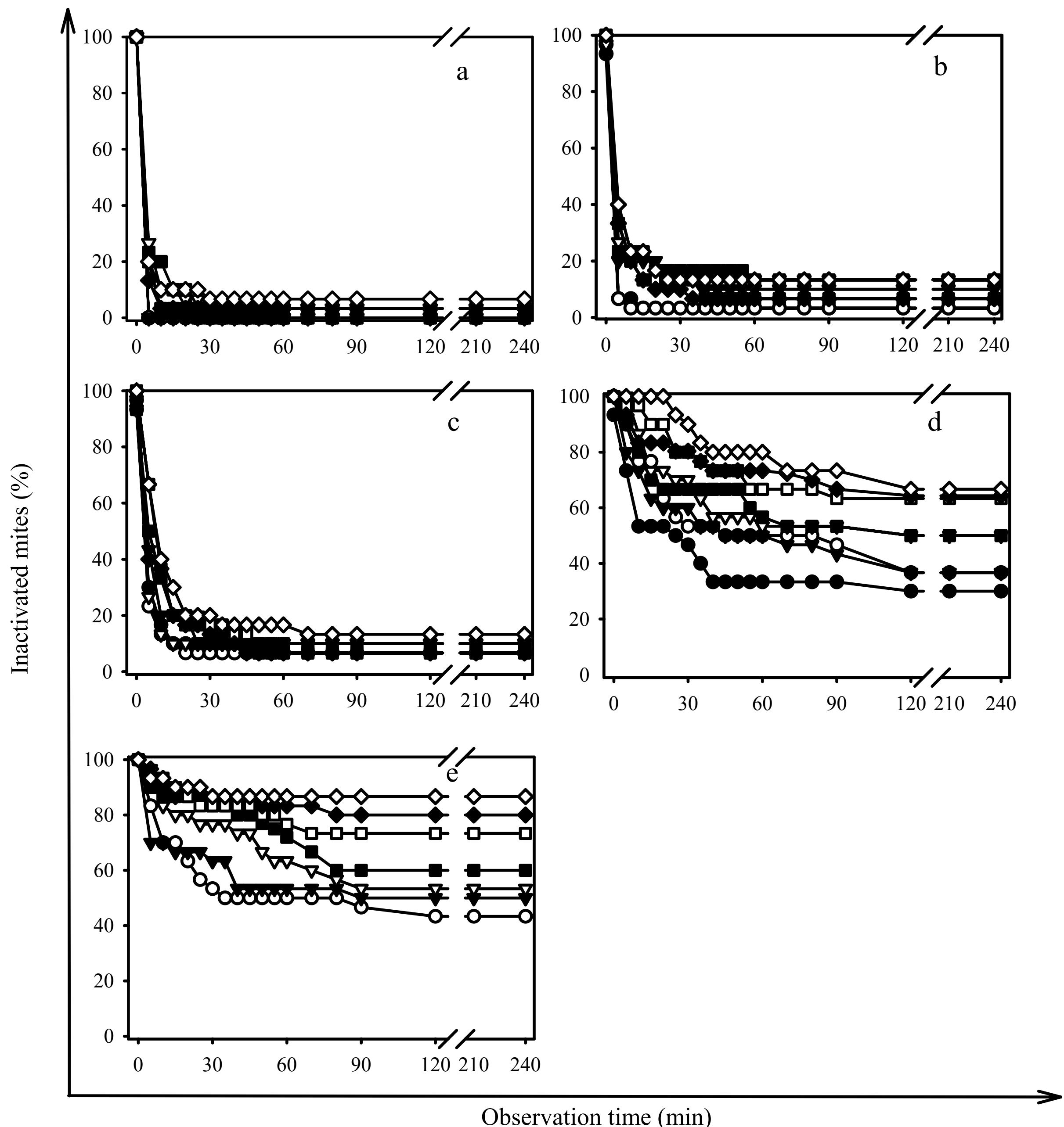

Fig. 3.1 Effect of contact time on the activity of *Varroa destructor* mites under treatment with 0.5% (a), 1% (b), 2% (c), 5% (d), and 7.5% (e) w/v propolis in 55% ethanol (solution B). Six mites per experiment, n = 5. ● - 5s, o - 10s, ▼ - 20s, ∇ - 30s ■ - 40s - 60s, ◆ - 75s, ◇ - 90s contact time. Even though the control treatments displayed narcosis of 53.3 to 100 % of the mites, directly dependent on the treatment time, narcosis lasted less than 5 min in all cases and, hence, was not incorporated in the graph.

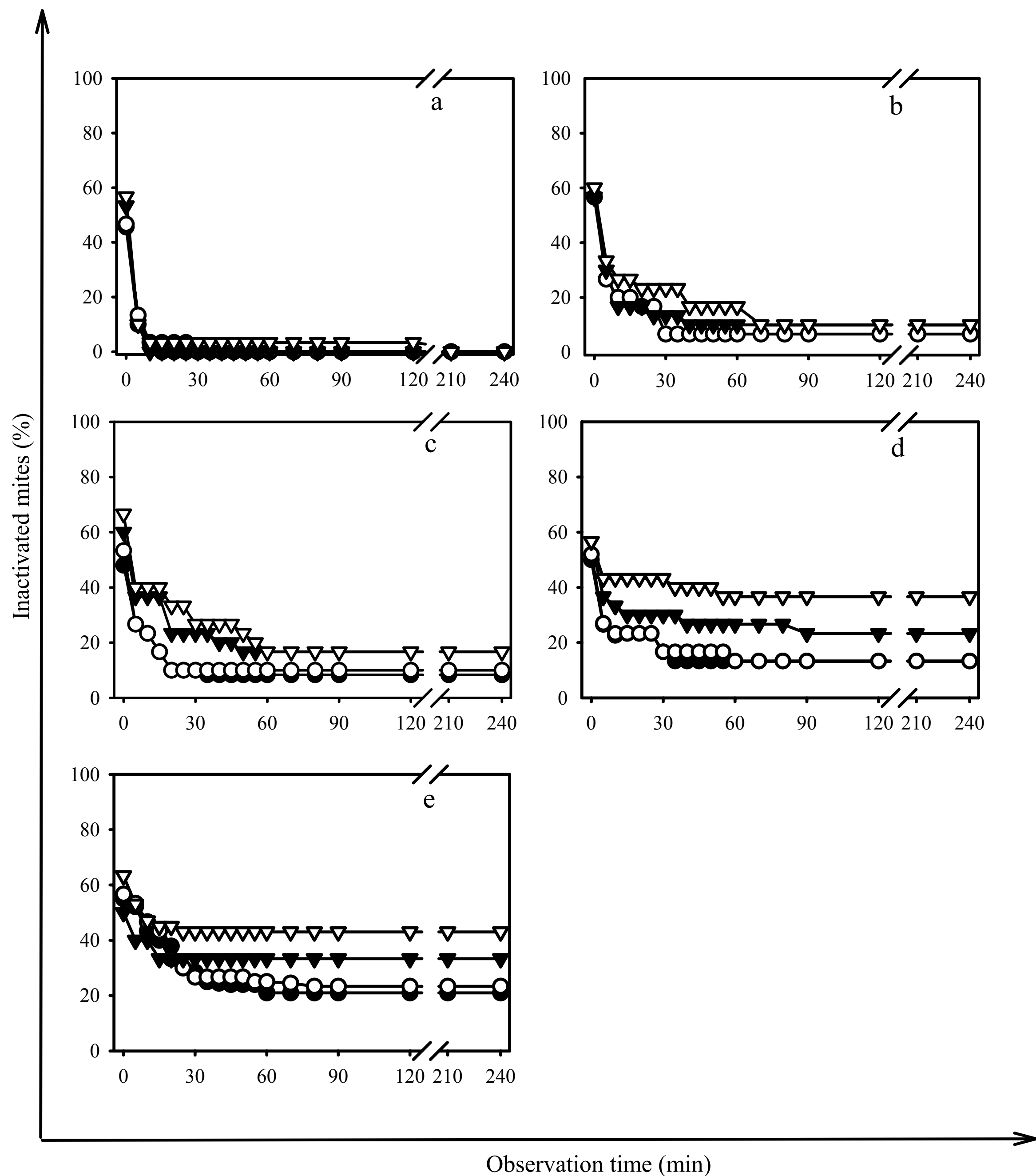

Fig. 3.2 Effect of contact time on the activity of *Varroa destructor* mites under treatment with 5% (a), 7.5% (b), 10% (c), 15% (d), and 20% (e) w/v propolis in 40% ethanol (solution A). Six mites per experiment, n = 5. ● - 20s, O - 40s, ▼ - 60s, ∇ - 90s contact time. Even though the control treatments displayed narcosis of 46% to 53 % of the mites, directly dependent on the treatment time, it lasted less than 5 min in all cases and, hence, was not incorporated in the graph.

Treatment of *V. destructor* mites with various propolis concentrations of solution B at different contact times showed that 100% narcosis was achieved for some minutes immediately after treatment, regardless of propolis concentration and contact time. Further observation of activity of the mites displayed that with lower concentration of solution B

(0.5%, 1% and 2%) narcosis lasted shorter (Fig. 3.1 a, b, c) and most mites recovered from narcosis in the first 5 to 15 minutes after treatment. Even if recovery was observed after this time it was very slow. Narcosis with higher concentrations of solution B (5%, 7.5%) lasted longer and most mites that could recover, recovered within the first 90 minutes after treatment (Fig. 3.1 d, e). Very few mites were observed recovering two hours after treatment. Treatment of *V. destructor* with solution A resulted in an initial narcosis of 46.7% to 53.3% (Fig. 3.2 a to e) which is lower in comparison with that of solution B. Most mites treated with lower concentration of solution A (5%) recovered within the first 10 min (Fig. 3.2 a), regardless of the contact time. Treatment time played a role in case of treatments with higher concentrations of propolis for both solutions A and B (Fig. 3.1 d, e, and Fig. 3.2 b, c, d, e). Treatment of mites with 10% (w/v) propolis in 55% ethanol (10% solution B) resulted in 100% mortality, regardless of the treatment time, indicating its high toxicity with the slightest contact time, as low as 5 s. As there was no activity of mites observed after treatment with 10% solution B it was not necessary to display this in Fig. 3.1.

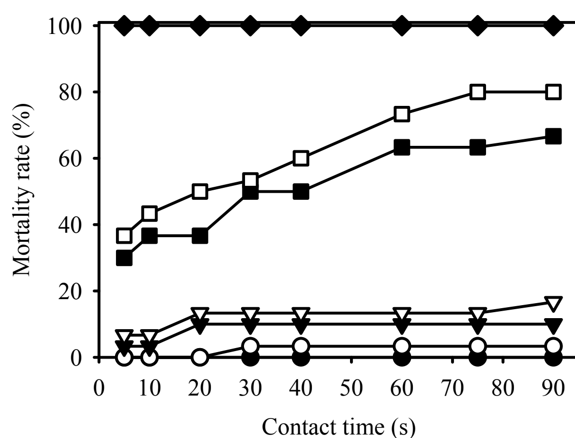

Fig. 3.3 Influence of contact time on the mortality rate of *Varroa destructor* under treatment with different concentrations (% w/v) of solution B (in 55% ethanol). Six mites per experiment, n = 5, percentages of the mean values are presented here. ●- control, ○- 0.5%, ▼- 1%, ▽- 2%, ■- 5%, □- 7.5%, ◆- 10% propolis. 4 h after treatment.

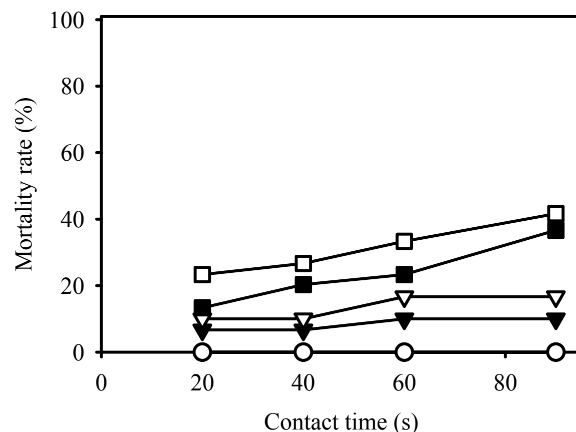

Fig. 3.4 Influence of contact time on the mortality rate of *Varroa destructor* under treatment with different concentrations (% w/v) of solution A (in 40% ethanol). Six mites per experiment, n = 5, percentages of the mean values are presented here. ●- control, ○- 5%, ▼- 7.5%, ▽- 10%, ■- 15%, □- 20%. The control experiment and 5% had no lethal effects, values overlapping at the x-axis. 4 h after treatment.

Even though some mites were observed recovering from narcosis, others could not recover at all. Mites that did not recover in the first 4 h after treatment were dead. This was

confirmed by incubating the treated mites for a total of 8 h. The effect of different propolis concentrations and contact times on the mortality rate of *V. destructor* is demonstrated in Fig. 3.3 and Fig. 3.4. The varroacidal action of propolis increased with increasing concentration and contact time except for lower concentrations of both solutions (Fig. 3.3, and Fig 3.4). Treatment of mites with 10% (w/v) solution B resulted in 100% mortality regardless of the treatment time, indicating its high toxicity with the slightest contact time. Treatment of mites with solution A, even with a concentration of 20% (w/v), resulted in a mortality rate less than 50% (Fig. 3.4).

Comparison of the specific heat production rates of Varroa mites before and after treatment with different concentrations of solutions A and B showed that even those concentrations that did not have a considerable effect on the mortality rate of mites dropped the specific heat production rate significantly (paired sample t-test, $p < 0.05$, to $p < 0.001$, Fig. 3.5 a and b). The reduction in the specific heat production rate due to treatment with propolis grew with increasing concentrations (Fig. 3.5 a and b insets), except at higher concentrations of propolis in 55% ethanol (solution B), where a saturation effect was observed (Fig. 3.5 b inset).

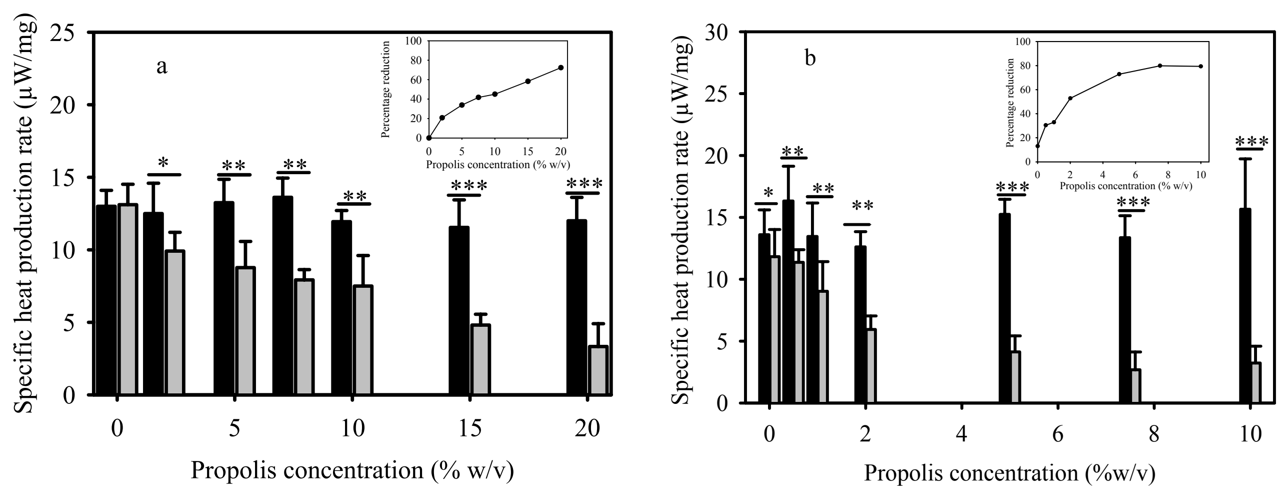

Fig. 3.5 Effect of different concentrations (% w/v) of propolis in 40% ethanol - solution A (a) and in 55% ethanol - solution B (b) on the mean specific heat production rate (mean ± S.D.) of *Varroa destructor* mites. 20 to 25 mites per experiment, n = 5. The inset in each graph, extracted from the corresponding graph, is a curve of percentage reduction of 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).

A feature common to all power-time (*p-t*) curves obtained after treatment with propolis, in addition to the reduction in the heat production rates, was the loss of the typical structures and subsequently smoothing of the curves.

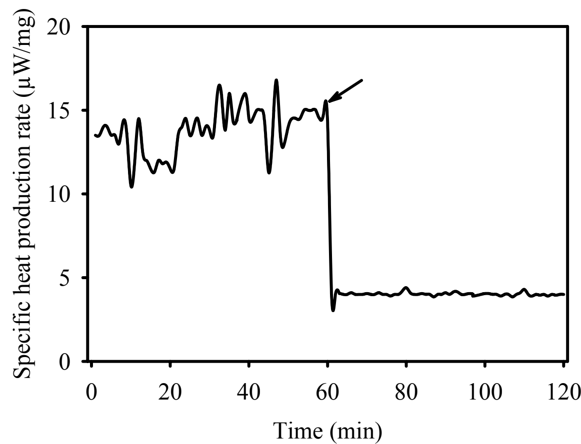

Fig. 3.6 Effect of treatment of *Varroa destructor* mites with 5% (w/v) propolis in 55% ethanol (solution B) on the structure and the level of the specific heat production rate (µW mg^{-1}) in a typical calorimetric experiment with 25 mites. After treatment with propolis, a period of 45 min (omitted in the graph) was required before starting to record the heat production rate again. The arrow indicates point of treatment.

The difference between the maximum and minimum points on the curves diminished progressively after treatment with increasing concentration of propolis. With treatments at higher concentrations of propolis the curves became nearly smooth, and lay at lower levels. Fig. 3.6 displays a typical example of a *p-t* curve whose structure was highly affected by the treatment with propolis.

3.5. Discussion

Treatment of mites with propolis causes narcosis and death. The length of narcosis and perhaps the subsequent death of mites after treatment with propolis concentration above a certain threshold depend on propolis concentration, solvent of extraction and length of contact time. This does not, however, hold true at lower concentrations, like in 0.5%, 1% and 2% solution B, or 5% of solution A. This is due to the fact that even if the length of contact time is prolonged, the concentration of bioactive components that penetrate into the mites' body is too low to cause considerable harm and, therefore, remains at a sublethal concentration. The length of contact time plays a role due to the fact that with the increase in the length of contact time, the amount of bioactive components penetrating into the mites' body increases, raising its concentration in the animal tissue, and achieving lethal doses. The slightest contact of Varroa mites with 10% solution B, regardless of the contact time, resulted in 100% mortality indicating that it is highly toxic. The narcotic effect of propolis on different animals has already been mentioned in the literature (Prokopovich et al. 1956, Prokopovich 1957). As seen from the present 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. The extraction of propolis in 70% ethanol enables the procurement of 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 control experiments of solutions A and B showed differences in the percentage of narcotised mites shortly after treatment, and in the reduction of heat production rates, there was no mite mortality observed in both cases. Unlike the controls, the treatments with different concentrations of the two propolis solutions showed considerable differences not only in their effect on narcosis and heat production rate, but also on the mortality rate of mites. This indicates that it is not the concentration of the alcohol, rather the difference in the ingredients of the two solutions of propolis that is responsible for the observed difference in mite mortality after the treatments with the two solutions.

Even though the treatment of mites with the different concentrations of solution A and weak concentrations of solution B (0.5%, 1%, and 2%) displayed only little varroacidal effects (Fig 3.3 and 3.4), it affected the specific heat production rate significantly (Fig. 3.5 a and b). As an example, treatment with 20% solution A with a contact time of 60 s killed only 33% of the mites, whereas the specific heat production rate was reduced by 75%. This is an indication that even the non-lethal or feebly lethal doses of propolis strongly emasculate the mites, and that the mites could not perform their locomotion nor were they able to move their body parts. The control experiments did not have lethal effects at all, but they affected the heat production rate. This fact suggests that monitoring the metabolic/heat production rates using calorimetric methods is more sensitive than observation of their locomotory activity, in order to judge the effect of propolis on mites. A limitation of the calorimetric method in the investigation of the action of propolis on Varroa mites was that the heat production rate during the recovery process from narcosis was difficult to follow. This difficulty arose due to the fact that the time required for the thermal equilibration of the calorimeter after opening it, to place the samples, was long (30 to 45 min). During this thermal equilibration time most of the mites have already recovered and attained a certain steady state metabolic rate. Had it not been for this limitation, the calorimetric method would have helped us to observe the heat production rate while the mites were narcotised, and in the gradual recovery process from narcosis

A feature common to almost all *p-t* curves obtained after the treatment with higher concentrations of propolis was the smoothness of the curve, and its position at lower level than before treatment. The highly structured *p-t* curves, with clearly distinct maximum and minimum signals were smoothed due to the treatment (Fig. 3.6), the extent of smoothing of the curve increasing with rising concentrations of propolis. This phenomenon may be explained as follows: all the mites may rest at a time, resulting in a minimum specific heat production rate, and all of them become very active simultaneously resulting in a maximum heat production rate. As an adaptation to their way of life the mites produce faeces (guanine) which is dry (Sammataro et al. 2000). Thus the deposition of faeces in the calorimetric vessel may not cause a considerable evaporational heat loss, and hence the troughs are mainly due to the simultaneous resting of mites. This feature of rest and simultaneous activity of the mites was observed outside the calorimeter, after placing them in a small glass vessel. Observation of their activity demonstrated that a mite rests for some time and starts moving again chaotically, disturbing the mites in the vicinity that respond in the same fashion. This process of chaotic activity lasts for some minutes and all the mites in the vessel may become very active, resulting in the maximum specific heat production rate. The phenomenon of rest and simultaneous activity is responsible for the structuring of the *p-t* curve. The smoothness of the *p-t* curve after treatment with propolis results due to the fact that the mites are highly weakened, and unable to perform their usual movement. They were able to move feebly or perform their resting and weakened metabolic activity, resulting in a *p-t* curve which is smooth.

The present experimental results showed that *V. destructor* mites are highly sensitive to propolis solution *in vitro*. It has been postulated (König and Dustmann 1988) that bees must be getting some benefit from the use of propolis in the beehive; otherwise they would not waste time and energy in collecting it. Thus, it is possible that propolis could affect Varroa mites in the beehive to a certain extent. The varroacidal action of propolis seems to be paradoxical, since propolis and Varroa mites are normally found in the beehive, the mites walking on thin propolis layers throughout the hive. The most plausible explanation as to why propolis does not kill Varroa mites in the beehive is that propolis is insoluble in the beehive interior. This is because most of the components of propolis are water insoluble. The water soluble components of propolis that could potentially affect mites in the beehive *in situ* comprise about 2.5% to 6.5%, based on the origin of propolis (Neunaber 1995). As seen in the case of efficacy (both narcosis and mortality) of solution A, where most of the water soluble and some water insoluble components are extracted, a higher concentration of propolis was

needed to observe a remarkable varroacidal effect. This fact displays that even if some of the components of propolis are dissolved in the high humidity in the hive's interior, their concentration is too weak to remarkably affect/kill the mites.

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

In order to reduce the amount of unnecessary chemicals of propolis in hive products the active varroacidal components of propolis may be fractionated and used separately. In addition to this, the synergistic action of propolis with essential oils, already being used as varroacides, may have to be investigated. If propolis is effective in the field experiment, and if it has no negative effect on the bees themselves, it may minimize the cost of beekeeping.

Propolis from different geographic origins differ from each other in their chemical makeup and, hence, probably in their Varroa narcotizing and varroacidal actions. Since the antivarroa action of propolis could be affected by temperature, the next two chapters will concentrate on the investigation of the antivarroa action of propolis from different geographic locations, at different experimental temperatures.