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Microcalorimetric investigation of the action of propolis on *Varroa destructor* mites

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Abstract

Treatment of *Varroa destructor* (Anderson and Trueman) mites with propolis has displayed both narcotic and lethal effects. The length of narcosis and rate of mortality showed dependence on the concentration of propolis used, the duration of contact time and the extraction procedure. Investigation of the action of propolis on the heat production rate of *Varroa* mites was performed by means of a Biocalorimeter (BCP 600). Calorimetric results point to the fact that even weak propolis concentrations with feeble lethal effects have a strong influence on the specific heat production rate. Thus, treatment with a 20% propolis solution in 40% ethanol resulted in a mortality rate of only 34% but dropped the heat production rate by 75%, a corresponding 5% propolis solution had no lethal effect but reduced the specific heat production rate still by 37% and treatment with a 0.5% propolis solution in 55% ethanol resulted in a non-significant mortality rate of 7% but reduced the specific heat production rate by 29%. Propolis showed a strong impact on the structure of the calorimetric power–time (p–t) curves. The usually highly structured p–t curves were smoothed due to treatment even with weak propolis concentrations.

Keywords: *Varroa destructor*; Microcalorimetry; Varroacides; Propolis; *Apis mellifera*

1. Introduction

Honeybee (*Apis mellifera* L.) colonies are opulent and unwavering habitat for a vast array of parasites and pathogens since host bees, honey, pollen and wax are present year round and the bees regulate the temperature and humidity within narrow limits [1]. One of such parasites that infest the western honeybee *A. mellifera* is the mite *Varroa destructor* (Anderson and Trueman) Oud. (Acari: Varroidae).

The infestation of *A. mellifera* by *V. destructor* reportedly originated nearly half a century ago [2–4] when the mites transmitted to *A. mellifera* colonies that had been introduced into the home range of *Apis cerana*, the mite's original host. *V. destructor* and *A. cerana* maintain a stable relationship, the latter having developed the proper mechanisms (for details see [5]) to check and maintain the population size of the former at a tolerable level. However, colony mortality of *A. mellifera* in temperate regions, unless treated, approaches 100% indicating that *V. destructor* has become one of the most serious threats facing both managed and feral honeybee colonies, beekeepers and the beekeeping industry as a whole [6]. Viral and bacterial infections are common within infested colonies, and may be transmitted by the mites or opportunistically invade weakened colonies [7,8].

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Since the introduction of *V. destructor* into Europe and the US, beekeepers have been trying to fight this pest with acaricides, chemicals that kill mites. Acaricides seemed to offer a solution to the problem of varroatosis even though their long-term application is associated with the accumulation of residues in bee products [9–13] and the emergence of resistant *V. destructor* strains [14–20]. To prevent or at least minimize the emergence of resistant *Varroa* strains, beekeepers and researchers should continue the search for new acaricides and the existing acaricides have to be used in rotation. Furthermore, the new acaricides should be, as much as possible, free of residue problems in hive products. Natural acaricidic products that are mixtures of different components with various modes of action may solve the problem of varroatosis [21] and the emergence of resistance against such acaricides may be unlikely or very slow. One of such natural substances that have a potential acaricidal action is propolis (bee glue).

Propolis is a resinous natural product collected by honeybees from various plant sources and stored in the beehive. The different components of propolis are produced by trees to avoid infection of injured tree parts, retard bud development during frost, protect pollen from being infected by microbes—insuring perpetuation of the species [22], and to help ward-off or kill insects or mite pests [23]. Apart from the purely mechanical uses of the glue-like and cementing properties of propolis in the blocking of holes and cracks, repairing of combs, strengthening the borders of the comb, making the entrance of the hive weather tight or easier to defend, varnishing the inner surface of the hive and minimizing water loss, its use in the beehive by the bees may also have a chemical basis [24]. Propolis has already displayed lethal action against several bacterial and fungal species, such as those pathogenic to honeybees [25,26]. The volatile components of propolis may reduce the microbial flora within the apiary. The chemical properties of propolis may have not only antimicrobial but also acaricidal value. Literature on the acaricidal, and even in general on the insecticidal properties of propolis is very limited. It has been suggested [27] that races of *A. mellifera* that prevail lower infestation rates by *V. destructor* may collect propolis, pollen and nectar whose components adversely affect the development of *Varroa* mites and thus guarantee lower infestation rates. The acaricidal action of propolis against *V. destructor* has been shortly mentioned in the literature [28] where the researchers have observed narcotic and lethal effects by smoking pieces of gauze used to cover the upper surface of frames in a beehive which were eventually propolised. A Petri dish assay of the insecticidal action of propolis, by allowing the mites to walk on a tissue paper moistened with propolis solution in a Petri dish, in our laboratory [29] showed that propolis has a strong narcotic and varroacidal action.

By employing the Petri dish assay method, one can judge the action of propolis as lethal or non-lethal—the two extreme ends of a continuous spectrum. Thus, the extent of weakening of the mites by the non-lethal doses of propolis is overseen or underestimated. In addition to that, in order to observe activity of the mites one may have to use a dissecting lens since mites are too small to detect feeble movements of individual body parts with the unaided eye and they may have to be prodded with a probe to see if they are dead or paralysed or alive [16,29], imparting subjectivity to the experimental results. In order to solve this problem, we have conducted calorimetric experiments to observe the kinetics of action of propolis on the metabolism of *Varroa* mites.

Calorimetry is a non-specific, non-invasive, and integrative method specially suited to monitor metabolism of living systems in all their existing forms without interference with the system [30]. Calorimetric methods have been employed in the investigation of drug effects on microbial cultures, such as well known antibiotics [31] and propolis [32], where these methods were shown to be superior to non-calorimetric methods. Microcalorimetry has also been applied in the investigation of insect growth and development [33–37] and effects of plant secondary metabolites on insects [38]. It has been pointed out [39] that terrestrial insects are the most frequently calorimetrically investigated small ''calorimeter sized'' animals since they are easy to gather, breed, keep, handle and measure in dry vessels without evaporation problems or high thermal inertia as opposed to aquatic organisms.

2. Materials and methods

2.1. Mite collection

Mites were collected from infested colonies in the garden of the Institute of Zoology, Free University of Berlin, Germany. The experiments were done in

summer 2000. Infested combs or pieces of combs containing drone broods were brought to the laboratory and adult *Varroa* females were collected from capped cells by opening and inspecting individual cells. In order to avoid starvation of the mites during the collection process collected mites were kept in a Petri dish on bee larvae or pupae. Collection of mites was done from both the larval and pupal stage of healthy drone brood, since mites at the different developmental stages of the brood did not show any significant difference in their sensitivity for pyrethroids in a laboratory bioassay [16]. Newly moulted adult mites, identified by their pale colour, relatively smaller size, and weak locomotion activities were excluded from the experiment since they may have a different response as hardening of the cuticle is still in progress. Mites, which seemed physically weak and abnormal and also those obtained from diseased brood were discarded.

Infested combs, taken out of the beehive and not needed immediately, were kept in an incubator at 34 ± 0.5°C until needed.

2.2. Determination of optimum temperature of metabolism

Various parasites have different mechanisms of adaptation in or on their hosts. Some parasites may have to lose some features or alter their optimum physiological requirements in order to fit into their host environment. One of such features may involve the compromise of optimum temperature of metabolism and growth. Since *Varroa* mites live in beehives where the temperature is controlled and maintained at about 34 °C by the honeybees, a question may arise as to whether the hive temperature is the optimum for *Varroa* mite metabolism or if the mite has compromised its optimum temperature as an adaptation for its parasitic lifestyle. To find out the optimum temperature of metabolism of the mites outside the beehive, calorimetric experiments were conducted at 20, 25, 30, 35, and 40 °C. The calorimeter used for this experiment was a Triflux calorimeter (THERMANALYSE TRI/11/78) of sensitivity 58 μV/mW and a vessel volume of 1.2 cm^3. After changing the temperature settings between experiments, the calorimeter was allowed to equilibrate for 24 h.

Ten to fifteen mites per experiment were weighed using a sensitive analytical balance (Sauter, Ebingen, Germany) and placed in the calorimetric vessel. The heat production rate was recorded for about 5 h. The experiment at each temperature setup was repeated five times and the results were presented as mean ± S.D. values.

2.3. Propolis extraction and preparation

Propolis samples used in our experiments were obtained from the research beehives in the garden of the Institute of Zoology, Free University of Berlin, Germany. In preparation for extraction, weighed and frozen propolis samples were homogenised using a coffee mill (type MZ Moulinex, France). Samples were frozen before homogenisation since unfrozen propolis, due to its sticky nature, does not lend itself to easy homogenisation. The homogenate powder was then suspended in 70 or 40% ethanol, in a ratio of 1:9 (w/v) for effective extraction [40], and extracted in a rotary evaporator (Rotationsverdampfer W-micro, Heidolf, Mannheim, Germany) at 60 °C for 2 h. After the allocated extraction time, the suspension was cooled at room temperature for about 1 h and suction filtered. The filtrate was dried in an incubator at 40 °C to weight constancy, which was achieved in 2 weeks time. The yield of extraction was 58% (w/w) for the extraction in 70% ethanol and 19% (w/w) in 40% ethanol.

The dried 70% ethanol extract was dissolved in 55% ethanol to make a 10% (w/v) stock solution of propolis. Even though 70% ethanol was used for extraction purpose, 55% ethanol was employed as a solvent 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 the 70% extract in 55% ethanol was brought into solution by agitation. The dried 40% ethanol extract was dissolved in 40% ethanol to make a 20% (w/v) propolis stock solution. The stock solutions were stored in a refrigerator for later use.

Different concentrations of propolis were prepared for the bioassay by diluting the stock solutions with ethanol of the concentration used to prepare the corresponding stock solution.

2.4. Petri dish bioassay

In order to observe the effect of contact time of different propolis concentrations on the mortality rate

of *V. destructor* mites, six mites per experiment were placed on a 3 cm × 3 cm single layer tissue paper in a Petri dish. The mites were treated by applying 400 μl of a given concentration of propolis on the tissue paper using an Eppendorf micropipette. The treatment was stopped after the allocated time by removing the mites from the Petri dish and placing them on a pad of paper towels for 1 min to blot the excess fluid on their surfaces. They were then transferred to a clean Petri dish and incubated at 34 ± 0.5°C for 4 h. At the end of the chosen time, their activity was observed under a dissecting lens by gently prodding them with a probe. The mites were then incubated for additional 4 h to see if there was a change in activity. Control experiments for each experimental group were run by treating the mites, for the corresponding treatment times, with 40 or 55% ethanol solution and also with distilled water.

An individual was considered dead if it showed no leg movement and/or movement of any other body part when gently prodded with a probe. If it showed movement, whether it was partially paralysed or normal, was counted as alive. Each treatment was repeated five times and the mean values were used in the presentation of results.

2.5. Calorimetric bioassay

As already pointed out, the Petri dish assay method enables one to tell whether a certain propolis concentration is lethal/narcotic or not. Apart from that, it does not help us in elucidating to what extent a certain concentration of propolis affects the metabolic rate of the mites and weakens them. With the intention of solving this problem, calorimetric experiments were conducted to observe the kinetics of action of different propolis concentrations on *Varroa* mites. The calorimeter used was a Biocalorimeter (BCP 600) with a sensitivity of 50 μV/mW and a vessel volume of 20 cm^3.

In order to compare the heat production rate before and after treatment, and to assess the impact of a certain concentration of propolis on the metabolic rate, 20–25 untreated mites per experiment were put in the calorimetric vessel and their heat production rate was recorded for 4 h. Recording was then stopped, mites removed from the calorimeter and treated with propolis. The treatments were done for 30 s with propolis in 55% ethanol and for 60 s with propolis in 40% ethanol. Ending up the experiment involved removing the mites from the damp tissue paper and putting them on a pad of dry tissue paper so that the moisture on their surface is blotted, which would otherwise interfere with the calorimetric signal considerably by elongating equilibration time of the calorimeter. The treated and blotted mites were put back in the calorimetric vessel and their heat production rate was recorded for 12–15 h. Recording of the heat production rate after treatment was run for longer period than before treatment since: (a) the moisture on surface of the mites, introduced due to treatment, interferes with the calorimetric signal due to evaporational heat loss and needs some time to diminish, (b) due to recovery from narcosis the mite's physiological activity may change through time after treatment until it reaches its ''after treatment steady state'' activity status. In the interpretation of results, only the part of the power–time (*p–t*) curves after the attainment of a steady-state heat production rate was considered. Each experiment was repeated five times and the mean ± S.D. values were used in the presentation of results. Control experiments for each experimental group were done by treating the mites for the corresponding time with 40 or 55% ethanol and also with distilled water.

3. Results

Specific heat production rates of the mites showed dependence on the experimental temperature, increasing sharply with the increase of temperature from 20 °C, the minimum experimental temperature used, to 30 °C (Fig. 1). With increasing temperature from 30 to 35 °C one could observe a gradual increase in the specific heat production rate. But the further increase of temperature from 35 to 40 °C resulted in a slight decrease in the specific maximum heat production rate and a slight increase in the specific minimum heat production rate, with the specific mean heat production rate remaining constant. At 20 °C, the minimum and mean specific heat production rates dropped below the baseline, being -1.9 ± 0.16 and -1.2 ± 0.26 μW/mg, respectively, whereas the specific maximum heat production rate was slightly above the baseline, being 0.35 ± 0.24 μW/mg.

Since the purpose of the calorimetric experiments was to observe the effect of non-lethal doses of

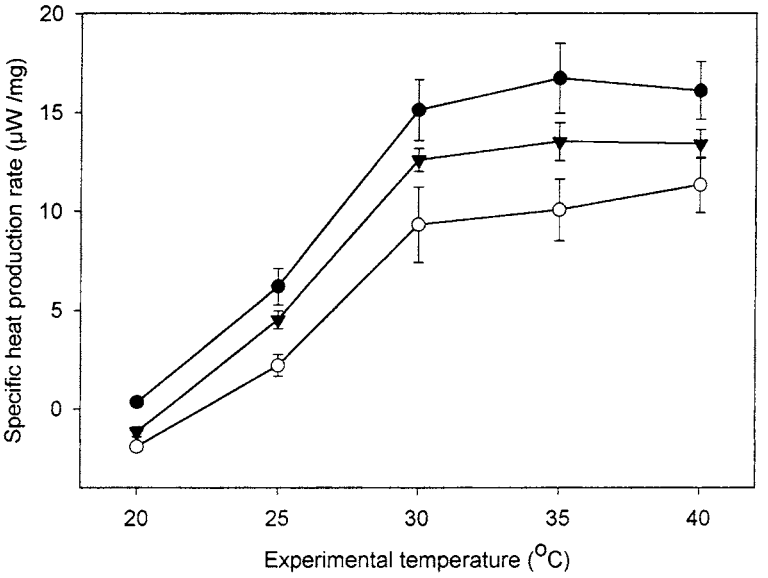

Fig. 1. Effect of temperature on the mass specific heat production rate of *V. destructor* mites, as represented by the specific maximal and minimal heat production rates derived from the maximal and minimal signals on the *p–t* curves, and the mean specific heat production rate, computed from the total heat production in the experimental time interval. Ten to fifteen mites per experiment, $n = 5$. Points on the curve represent mean ± S.D. values. Maximum (●), minimum (○), and mean specific heat production rates (▼).

propolis at non-lethal contact times, it was first of all, essential to elucidate effect of the length of contact time of different propolis concentrations on the mortality rate of mites. As can be seen from Fig. 2, mortality rate increased with increasing contact time for propolis concentrations of 5 and 7.5% in 55% ethanol. But the lower concentrations of propolis used (0.5, 1, and 2%) did not show much increase in

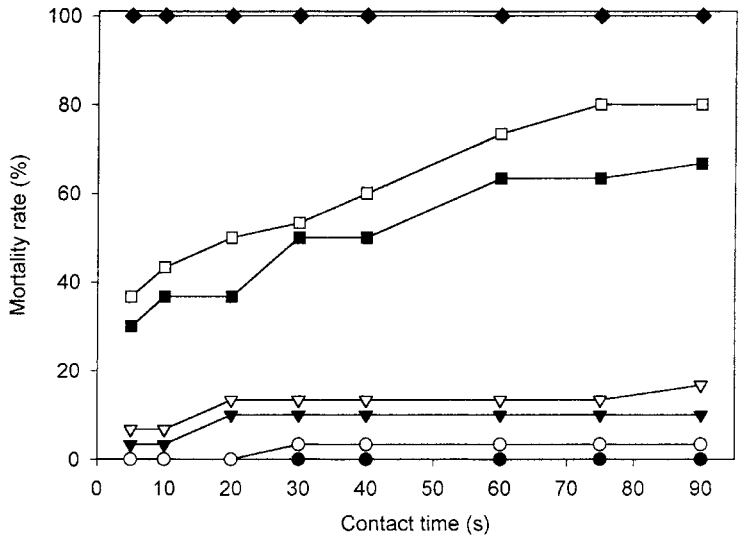

Fig. 2. Effect of contact time of different propolis concentrations (% w/v) in 55% ethanol on the mortality rate of *V. destructor* mites. Six mites per experiment, $n = 5$. Control (●), 0.5% (○), 1% (▼), 2% (▽), 5% (■), 7.5% (□), and 10% (◆) propolis.

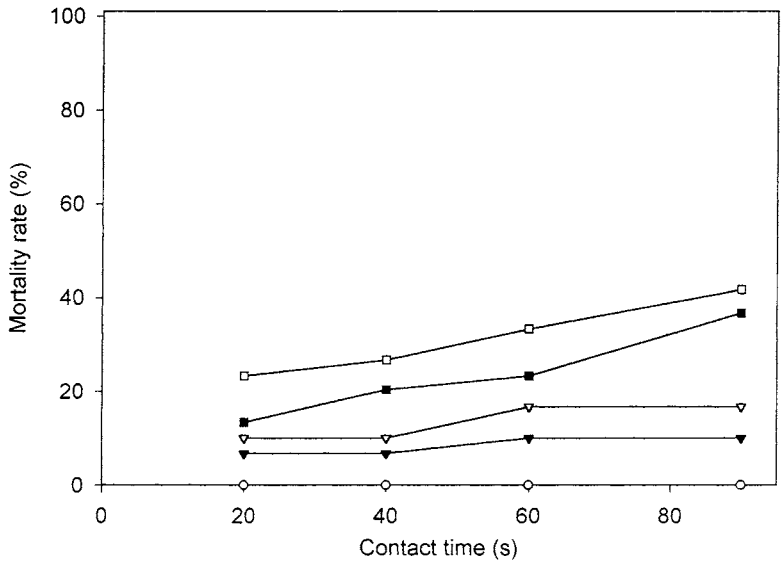

Fig. 3. Effect of contact time of different propolis concentrations (% w/v) in 40% ethanol on the mortality rate of *V. destructor* mites. Six mites per experiments, $n = 5$. Control (●), 5% (○), 7.5% (▼), 10% (▽), 15% (■), and 20% (□) propolis. The control experiment and 5% propolis have no lethality effects, values overlapping at zero (the filled-and the blank circles overlap at the *x*-axis; only blank circles are shown).

lethality effect with increasing contact time. In contrary to these the shortest contact time, for 5 s, with 10% propolis in 55% ethanol resulted in 100% mortality. In the case of different propolis concentrations in 40% ethanol, mortality rate, even though it was weak, seemed to increase with increasing contact time and concentration (Fig. 3).

For the calorimetric experiments, a contact time of 60 s was employed for propolis in 40% ethanol since the mortality rate at this contact time was not very

Table 1
Percentage reduction in the mean and maximum specific heat production rates, *p*, and mortality rate of *V. destructor* mites by different propolis concentrations

Propolis concentration (%, w/v)	% Reduction in mean *p*	% Reduction in maximum *p*	Mortality rate
In 40% ethanol (60 s treatment)			
20	73.0	69.5	33.7
15	59.6	59.6	23.6
10	47.2	48.0	16.9
7.5	44.9	44.0	10.7
5	37.1	34.8	0.0
2	24.7	19.1	0.0
Control	0.0	7.9	0.0
In 55% ethanol (30 s treatment)			
10	79.8	82.0	100
7.5	78.6	78.7	54.0
5	71.9	69.0	49.4
2	52.8	57.0	14.6
1	32.6	31.5	11.2
0.5	29.2	29.0	7.0
Control	12.9	20.0	0.0

high. However, since a contact time of 60 s resulted in a high mortality rate in case of 5, 7.5 and 10% propolis in 55% ethanol, a contact time of 30 s was used for experiments with propolis dissolved in 55% ethanol.

Comparison of the mean and maximum specific heat production rates on one hand, and mortality rate, from the Petri dish assay experiment on the other hand, displayed that even those propolis concentrations that

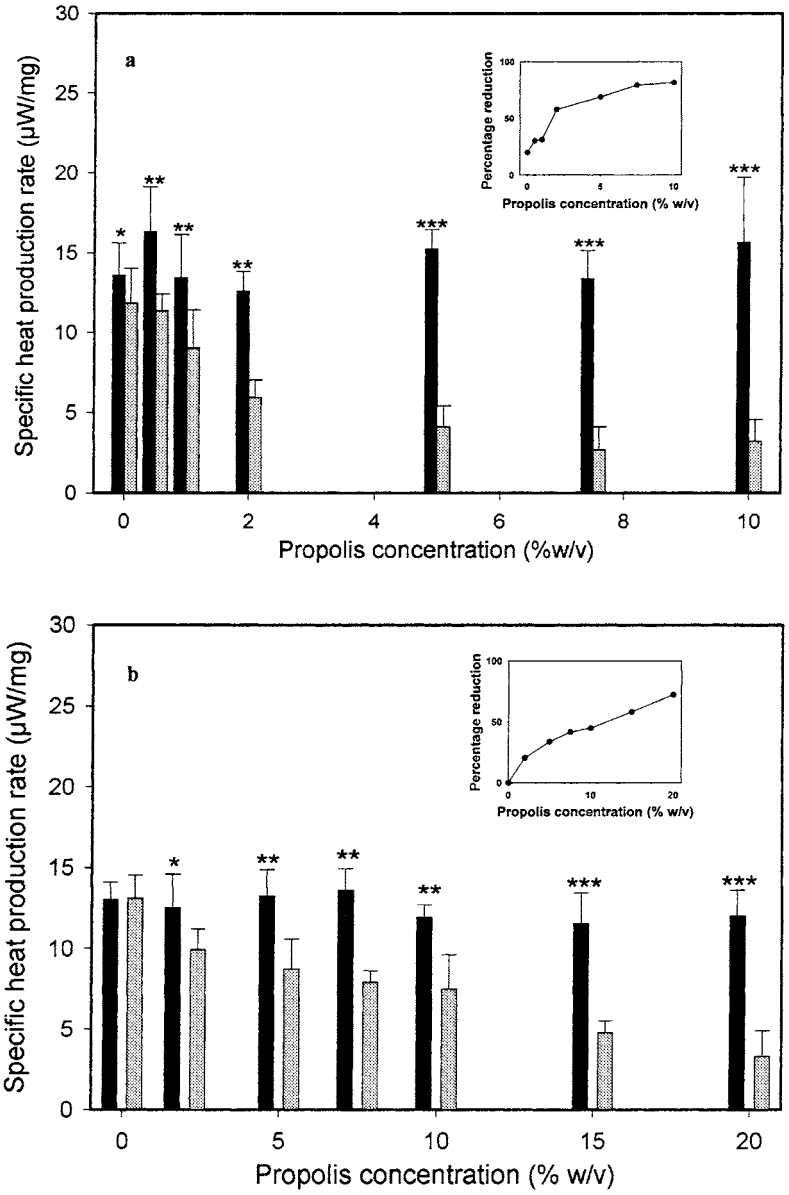

Fig. 4. Effect of different propolis concentrations (% w/v) in 55% ethanol (a) and 40% ethanol (b) on the mean specific heat production rate (mean ± S.D.) of *V. destructor* mites. Twenty to twenty five mites per experiment, $n = 5$. The inset in each graph, extracted from the corresponding graph, is a curve of 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: $(^{*})$ $P < 0.05$; $(^{**})$ $P < 0.01$ and $(^{***})$ $P < 0.001$ (paired sample t-test).

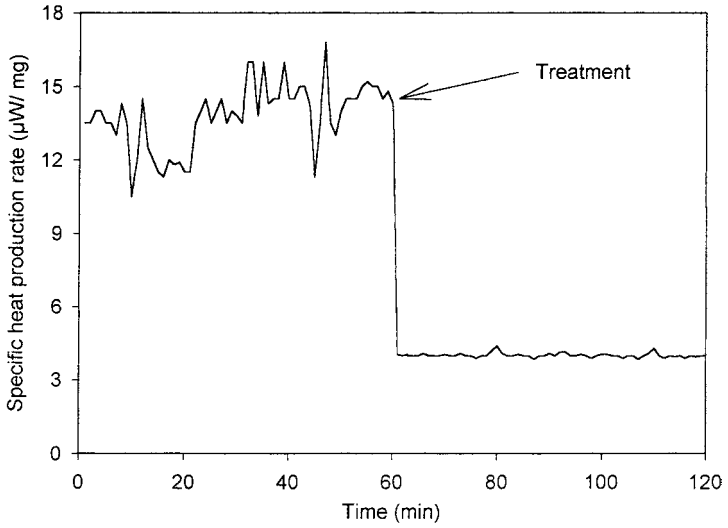

Fig. 5. Effect of treatment of *V. destructor* mites with 5% (w/v) propolis in 55% ethanol on the structure and the level of the specific heat production rate (μW/mg) in a typical calorimetric experiment with 25 mites. After treatment with propolis, a period of 45 min (omitted in the graph) was required before starting to record the heat production rate again.

did not show considerable effects on the mortality rate had a strong influence on the heat production rate (Table 1). The reduction in the mean specific heat production rate due to treatment with a certain propolis concentration was comparable with the reduction in the maximum specific heat production rate except for the control experiments, where the latter was found out to be more sensitive than the former. The treatment with 10% propolis in 55% ethanol resulted in 100% mortality, but still some amount of heat production was detectable.

The reduction in the mean specific heat production rate due to treatment with propolis grew with increasing propolis concentrations (Fig. 4), except at higher concentrations of propolis in 55% ethanol, where probably a saturation effect was observed.

A feature common to all the *p–t* curves obtained after treatment with propolis was the smoothing of the curve. The difference between the maximum and minimum points on the curve, after treatment with propolis, diminishes with increasing concentration of propolis. With treatments at higher concentrations of propolis, the curves become nearly smooth. Fig. 5 displays a typical example of a *p–t* curve whose structure was highly affected by the treatment with propolis.

4. Discussion

The optimum temperature of metabolism of *V. destructor* mites lies within the range 30–40 °C, the temperature of the beehive. This indicates that the parasitic life style of the mite did not lead to a compromise of its optimum temperature of metabolism. With the increase of temperature from 35 to 40 °C, a slight decrease in the maximum as well as an increase in the minimum specific heat production rate was observed, but the mean specific heat production rate remained nearly constant. This phenomena may be explained as follows: at around 35 °C, all the mites may rest at a time, resulting in a minimum specific heat production rate, and all of them may become very active simultaneously resulting in a maximum heat production rate. This feature of rest and simultaneous activity of the mites was also observed outside the calorimeter, after putting them in a small glass vessel. Observation of their activity witnessed 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* curves. At 40 °C, probably the environment was inconvenient and made the mites restless, moving continuously—resulting in a higher minimum specific heat production rate, reducing the difference between the maximum and minimum points on the *p–t* curves, i.e., smoothing the curve. At 20 °C, the minimum and mean specific heat production rates dropped below zero, this probably shows that the amount of heat produced at this temperature was lower than the amount of heat lost in the evaporation of fluids; but whether the mites produce much amount of fluid excreta at lower temperatures than at higher temperature is not clear.

Treatment of the mites with propolis showed both narcosis and death. The narcotic effect of propolis on different animals has been mentioned already in the literature [41,42]. Previous experimental results in our laboratory [29] proved that propolis has a strong narcotic and lethal effect on *V. destructor* mites and that the length of narcosis depended on propolis concentration, solvent of extraction and length of contact time. A limitation with 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 temperature equilibration of the calorimetric interior after opening is long (30–45 min). During this 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.

The mortality rate of *Varroa* mites due to contact with propolis, as seen from Figs. 2 and 3, depended on concentration, contact time and the solvent of extraction. Propolis extracted in 70% ethanol was superior to that extracted in 40% ethanol. The slightest contact of *Varroa* mites with 10% propolis in 55% ethanol, regardless of the contact time, resulted in 100% mortality indicating that it is highly toxic. In the case of treatments with lower concentrations of propolis, 0.5, 1, and 2% propolis solutions in 55% ethanol, and 5, 7.5, and 10% propolis solutions in 40% ethanol, the mortality rate seemed not to grow much with the increase in contact time, indicating the limited efficiency of these propolis concentrations. But mortality rate of *Varroa* mites rose with increasing contact time with 5 and 7.5% propolis in 55% ethanol, and 15 and 20% propolis in 40% ethanol.

Even though the treatment of mites with propolis in 40% ethanol and weak concentrations of propolis (0.5, 1, and 2%) in 55% ethanol displayed only little varroacidal effects, it affects the heat production rate significantly (Table 1), indicating that even the non-lethal or feebly lethal doses of propolis emasculate the mites strongly. The control experiments did not have lethal effects at all, but they affected the heat production rate slightly, the maximum specific heat production rate affected more than the mean one. This may show that, in order to detect the influence of very weak concentrations of varroacidal agents on the metabolism of *Varroa* mites, the maximum peaks are more sensitive than the mean peaks.

A feature common to almost all *p–t* curves obtained after the treatment with propolis was the smoothness of the curve. The highly structured *p–t* curves, with clearly distinct maximum and minimum signals were smoothed due to the treatment (Fig. 5), the extent of smoothing of the curve increasing with rising concentrations of propolis. This smoothing of the curve may be due to the fact that the mites were highly weakened and could not perform their usual chaotic movement.

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 probable explanation for the question why propolis does not kill *Varroa* mites in the beehive is that propolis in general and most of its components in particular, are water insoluble in the beehive's interior. The water-soluble components of propolis comprise about 2.5–6.5% only depending on the origin of propolis [43]. One needs a higher concentration of propolis to observe a remarkable varroacidal effect when propolis was extracted and used in 40% ethanol, where most of the water-soluble and some water-insoluble components were extracted. This shows that even if some of the components of propolis are solubilised in the high humidity of the hive's interior, their concentration is too weak to kill the mites. Most of the varroacidal agents of propolis are

water insoluble leading to the superiority of propolis extracted in 70% ethanol as compared to propolis extracted in 40% ethanol.

Even though 10% propolis in 55% ethanol resulted in 100% mortality, the heat production rate did not drop to the base line as would have otherwise been expected. The systemic microbes having already started decomposition of the dead body and may contribute this heat production.

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