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Thermochimica Acta 399 (2003) 171–180

thermochimica acta

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Microcalorimetric and respirometric investigation of the effect of temperature on the antivarroa action of the natural bee product-propolis

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Received 7 June 2002; received in revised form 30 July 2002; accepted 9 August 2002

Abstract

The antivarroa action of propolis and its synergism with temperature was investigated calorimetrically and respirometerically, using female *Varroa destructor* mites from adult workers, worker- and drone broods.

The treatment of *Varroa* mites with 4% propolis affected their metabolic activity, with the influence directly related to the temperature of treatment. The changes in heat production and oxygen consumption rates, as a function of temperature, showed similar patterns before as well as after treatment with 4% propolis.

The mites collected from worker- and drone broods reacted similarly to propolis treatment at different temperatures. In contrast, the mites from adult workers, phoretic mites, responded differently: the treatment with 4% propolis at 40 °C resulted in 100% mortality of mites from adult workers but only reduced the heat production rate of mites from worker- and drone broods by 68 and 60%, respectively.

Exposure of mites to 45 °C agitates them as witnessed by the elevated heat production rates (23.5 ± 2.5 μW/mg compared to that at 35 °C with 14.4 ± 1.0 μW/mg). After treatment with propolis at 45 °C all mites died regardless of their origin indicating that the simultaneous use of varroacides and high temperature treatment for a short period of time could be more effective rather than the prolonged use of either method.

Keywords: *Varroa destructor*; *Apis mellifera*; Propolis; Calorimetry; Respirometry; Varroacid

1. Introduction

Varroa destructor (Anderson and Trueman) is a serious ectoparasitic pest of the western honeybee *Apis mellifera* L., infesting both feral and managed colonies. It has caused the destruction of numerous colonies and the subsequent reduction in the number of beekeepers and production of honey and other bee products in different parts of the world after its accidental introduction as a pest in the last three decades [1–7]. To save their colonies from obliteration beekeepers are using acaricides as short-term solutions. But the use of chemical acaricides is not free from drawbacks: accumulation of residues in bee products [8–12], hazards to the bees and/or the beekeeper [13] and the development of acaricide resistance [14,15].

There are ever increasing numbers of reports of the resistance of *V. destructor* against various acaricides

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0040-6031/02/$ – see front matter
PII: S0040-6031(02)00453-7

in different parts of the world: fluvalinate [15–21], coumaphos [22,23], bromopropylate and chlordimeform [24] and to amitraz [25]. These problems associated with the use of acaricides provide incentives to bee researchers and beekeepers to search for better acaricides or methods to control *Varroa* mites. Different methods, like the biotechnical mite control [26,27], heat treatment of the brood using a Mitezapper [28] and the infested adult workers [29], a combination of biotechnical control and acaricide treatment [30,31], a combination of biotechnical control and heat treatment [32,33] have been shown to be effective in the control of *Varroa* mites. Of particular interest in the search for new acaricides are compounds that are natural in origin. Botanical extracts and essential oils have exhibited some efficacy as means to control *Varroa* mites [13,34]. Many plants produce essential oils or other chemicals that are used as natural pesticides to ward off insect herbivores or prevent infection of wounds. One of such groups of chemicals produced by plants is propolis.

Propolis is a complex mixture of several compounds collected by honeybees from plants, mixed with wax and some enzymes of the bee's salivary glands and accumulated in the beehive for the construction or protection of the hive [35]. A laboratory assay of the varroacidal action of propolis [36] displayed that it possesses both narcotic and lethal action with 10% propolis solution killing 100% of *Varroa* mites even at a very short contact time of 5 s. Lower concentrations display varying degrees of suppression of the metabolic rate of the mites.

In our present experiments we will try to demonstrate how experimental temperature affects the varroacidal and *Varroa*-weakening action of propolis on *Varroa* mites from different castes and developmental stages of the honeybee *Apis mellifera carnica* using calorimetric and respirometric experiments. The results of our experiments may elucidate if the varroacidal action of propolis can be augmented by a simultaneous exposure of the mites to higher or lower temperature extremes to reduce the treatment time and/or increase the efficacy.

Comparison of respirometric and calorimetric results at different ambient temperatures may enable us to evaluate the reliability of the simpler and inexpensive respirometry in the absence of the more precise, direct and expensive calorimetry.

2. Experimental

2.1. Animal material

Infested honeybee colonies of the bee race *A. mellifera carnica* from the research beehives of the Institute of Zoology, Free University of Berlin, Germany, were used as sources of *V. destructor* mites for our present experiments. The experiments were done in summer 2001. At the beginning of autumn of the previous year the experimental colonies were treated with formic acid one time to reduce the infestation level and eventual annihilation of the colony by *Varroa* mites.

Female *V. destructor* mites were collected from adult workers, worker brood and drone brood. The collection of mites from the brood stage was done at room temperature by opening and inspecting healthy brood. During the collection process mites were kept in a Petri dish on a corresponding bee pupae in order to avoid starvation. Newly moulted adult mites were excluded from the experiment since they may be possible sources of error as development of the cuticle could still be in progress. Mites that seemed weak and abnormal were discarded. Collection of mites from adult workers was done by very cautiously dislodging them from the surface of the bees with the help of a blunt needle. Though this collection process was very time consuming and tedious, it is a safe method as far as it is done cautiously.

2.2. Calorimetric experiments

The calorimetric experiments were performed using two isothermal calorimeters: (i) a Biocalorimeter B.C.P. (Messgeräte Vertrieb, München, Germany) with a sensitivity of 45 μV/mW and a vessel volume of 12 cm^3 and (ii) a Thermanalyse Calorimeter (Messgeräte Vertrieb, München, Germany) of a sensitivity 45 μV/mW and a vessel volume of 15 cm^3. The calorimetric vessels are big enough to provide adequate oxygen for the entire experimental period. The calorimetric experiments were done at temperatures of 25, 30, 35, 40, 45 and 50 °C each with the mites collected from adult workers, worker brood and drone brood.

Twenty to 30 mites were weighed before each experiment using a sensitive analytical balance (Sauter, Ebingen, Germany), transferred into the calorimeter and the heat production rate was recorded for 2–3 h.

In case of experiments with 45 °C the heat production rate before treatment was recorded only for 45 min since the mites started dying within 90–180 min after exposure. After the pre-selected time recording was stopped, mites removed from the calorimeter and weighed again immediately to find out the change in weight. From this one may evaluate the rate of utilization of reserve food under starving condition and consequently elucidate the amount of haemolymph the mites could utilize from their host to maintain their weight and physiological status under natural and non-starving conditions. After weighing the mites were immediately treated with a solution of 4% propolis in 55% ethanol as described below. Having blotted the excess fluid from their surface the mites were weighed again to obtain the after treatment initial weight, put back into the calorimeter and the heat production rate was recorded further for 3–5 h. The mites were weighed at the end of the calorimetric experiment to determine the rate of weight change. The weight loss of a mite per day was extrapolated from the weight loss during the experimental periods. In this respect the rate of weight change is presented as percentage wet weight loss per mite per day before and after treatment with 4% propolis at the different experimental temperatures.

2.3. Treatment of mites with propolis

Since the goal of these experiments was to observe the effect of temperature on the antivarroa action of a non-lethal dose of propolis, a 4% propolis in 55% ethanol [36] was used. It made no sense to apply a lethal dose of propolis since calorimetry after treatment with such doses is irrelevant. The propolis used for these experiments was obtained from Holeta Bee Research Center, Ethiopia. It was extracted in a rotational evaporator (Rotationsverdampfer W-micro, Mannheim, Germany) for 2 h in 70% ethanol. The dried extract was dissolved in 55% ethanol for further use.

In preparation for treatment the mites were put in a clean Petri dish on top of a 3 cm × 3 cm tissue paper (Kimwipes® Lite 200, Kimberly-Clark®). Treatment of the mites was done for 30 s by applying 250 μl of the 4% propolis solution on the tissue paper, not directly on the mites. The treatment was ended up after the allocated time by removing the mites from the Petri dish and placing them on a pad of paper towel for 1 min, to blot the excess fluid on their surfaces. Blotting of the excess fluid from the mites' surface after ending up the experiment was important since it otherwise would interfere with the calorimetric signal due to evaporational heat loss [37]. The treated and blotted mites were weighed again, placed back into the calorimetric vessel and their heat production rate recorded. Control experiments for each experimental group were done by treating the mites with 55% ethanol and also distilled water.

2.4. Respirometric experiments

The effect of temperature and propolis treatment on the oxygen consumption rate of *V. destructor* mites from drone brood was investigated at 25, 30, 35, 40, 45 and 50 °C using manometric methods. The respiration experiments were done using Warburg vessels of volume about 12 ml and 50–60 mites per experiment. CO_2 produced during respiration was absorbed by a 4% KOH solution. In order to avoid access of the mites to the KOH solution the opening to the side arm was fitted with a very thin layer (1 mm thick) of porous spongy material with a pore size of ca. 0.9 mm (a small piece of cotton can also be used for this purpose) that allows air but not the mites to enter the arm. Recording the oxygen consumption rate was started after a temperature equilibration time of 30 min and further recording was done in intervals of 30 min, for 2 h before treatment. The experimental time for each temperature set-up was equivalent to the calorimetric experimental times mentioned above. Each measurement was done fivefold, but ninefold in case of measurements with 45 °C since the experimental time for this temperature set-up was short compared to the other temperature set-ups due to mite death with prolonged experimental period.

Finally comparison of the effect of propolis at different experimental temperatures on the metabolic rate of mites from the mentioned developmental stages will be made to see if the mites have different responses.

2.5. Statistical analysis

Results were presented as mean ± s.d. values. The level of difference in the heat production rate, oxygen consumption rate and weight loss rate at the different

experimental temperatures, before and after treatment with propolis was determined using the paired sample *t*-test and a critical value of $\alpha = 0.05$.

3. Results

Mites obtained from a dult workers, worker brood and drone brood died immediately after exposure to 50 °C in both calorimetric and respirometric experiments. Hence the results at this temperature set-up are missing in most graphs since the rates are nil.

Mites collected from worker- and drone broods have comparable specific heat production rates at different experimental temperatures, except at 45 °C where the mites from worker brood exhibited a significantly higher heat production rate. At 25 °C mites collected from adult workers showed a significantly higher specific heat production rate: $8.4 \pm 1.4\,\mu$W/mg, as compared to that produced by mites from worker- and drone broods, which amounted to $5.0 \pm 0.4\,\mu$W/mg and $6.1 \pm 1.2\,\mu$W/mg, respectively (Fig. 1). These groups of mites also had a significantly higher specific heat production rate at 35 °C compared to the other two groups. But at 30 and 40 °C there was no significant difference. Regardless of where the mites originate from, heat production rates increase with raising calorimetric temperature achieving constant rates between 35 and 40 °C. With the shift of temperature from 40 to 45 °C the heat production rate grows drastically, e.g. from a value of 14.6–23.5 μW/mg in case of mites collected from worker brood. The high heat production rate at this elevated temperature value lasted for a short period of time: 90–180 min. After this time interval the curve declines due to death of mites, which ensues faster in case of *Varroa* mites from adult workers than of those from worker- and drone broods.

Treatment of *V. destructor* mites with 4% propolis resulted in a reduction in the heat production rate. The extent of reduction increased with the experimental temperature especially in case of mites obtained

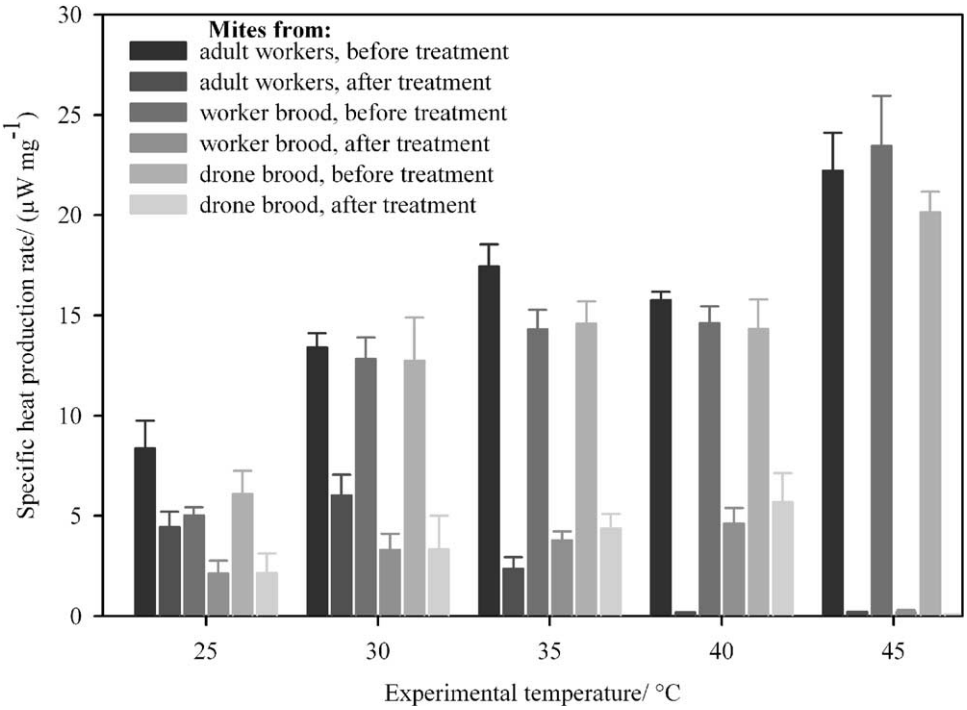

Fig. 1. The effect of temperature on the specific heat production rate of *V. destructor* mites before and after treatment with 4% propolis. Twenty to 30 mites per experiment, $n = 5$, mean ± s.d. The treatment with 55% ethanol (control) reduced the heat production rate by 5–9% regardless of temperature and origin of mites.

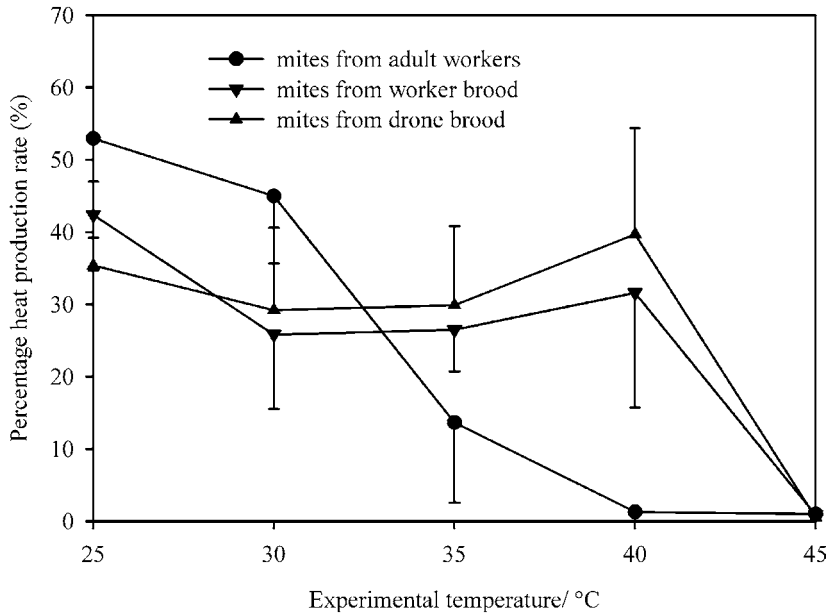

Fig. 2. Effect of temperature on the percentage residual heat production rate (p after treatment/p before treatment × 100) of *V. destructor* mites after treatment with 4% propolis. Twenty to 30 mites per experiment. $n = 5$, mean ± s.d. After treatment with 55% ethanol (control) the residual heat production rate lay between 91 and 95% regardless of the experimental temperature and origin of mites.

from adult workers (Fig. 2). They were all dead after treatment with 4% propolis at 40 °C whereas those from worker- and drone broods showed a reduction in the heat production rate by 68 and 60%, respectively. Regardless of the origin of mites, 100% mortality was achieved after treatment with 4% propolis at 45 °C, the heat production rate dropping to the base line (Figs. 1 and 2). *Varroa* mites from worker- and drone broods showed a nearly similar response to treatment with propolis at different experimental temperatures, whereas *Varroa* mites from adult workers have a different response (Fig. 2). The control experiments, treatment with 55% ethanol rendered a reduction in the specific heat production rate by 8–11% regardless of the temperature of treatment.

Heat production and oxygen consumption rates of *Varroa* mites from drone brood behaved similarly before and after treatment with 4% propolis at different experimental temperatures (Fig. 3). With the increase of temperature by 10 K from 25 to 35 °C the specific heat production rate (Q_{10}) before treatment increased by a factor of 2.4, the corresponding oxygen consumption rate by a factor of 2.3. Considering the temperature interval between 30 and 40 °C where the metabolic rate is nearly constant, the heat production rate increased by a factor of 1.1 only whereas the oxygen consumption rate grew by 1.2. After treatment with 4% propolis the change in the heat production rate and oxygen consumption rate showed a different pattern than before treatment. With the temperature increase from 25 to 35 °C the heat production rate changed by a factor of 2.0 whereas the oxygen consumption rate by 1.5. The Q_{10} value after treatment with 4% propolis for the transition from 30 to 40 °C amounted to 1.7, the oxygen consumption rate to 1.5 (Table 1). The treatment with 55% ethanol (control experiment) reduced the oxygen consumption rate by 6–10% independent of the experimental temperature. The other control experiments showed no effect.

Utilization of own reserve food by *Varroa* mites, displayed by the loss of wet weight during starvation, is highly affected by the experimental temperature. The mites collected from adult workers lose a higher proportion of their body weight per starvation hour than those from drone- and worker broods. A mite from adult workers may utilize 1.5-fold of its own

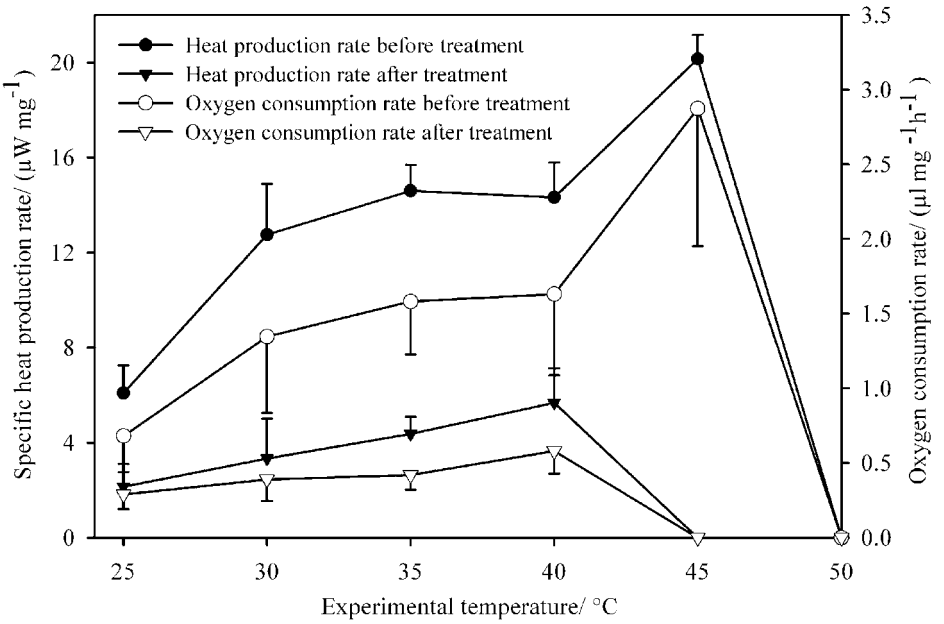

Fig. 3. Effect of temperature on the specific heat production rate and oxygen consumption rate of *V. destructor* mites from drone brood before and after treatment with 4% propolis. Twenty to 30 and 50–60 mites per experiment for the calorimetric and respirometric experiments, respectively. $n = 5$ (but $n = 9$ for the respirometric measurements at 45 °C), mean ± s.d. The oxygen consumption rate is reduced by 5–11% and the heat production rate by 5–9% in the control group.

weight per day at 25 °C, whereas that from worker- or drone broods could utilize 1.2- or 1.1-fold of its weight, respectively. The first value is significantly different from the latter two, which do not display significant difference among each other. At 45 °C a mite from adult worker, worker brood or drone brood could utilize 4.7-, 3.0- or 3.3-fold of its weight per day, respectively (Fig. 4). Percentage of wet weight of a mite utilized by itself after treatment with 4% propolis declined drastically with increasing temperature, especially in case of mites from adult workers. After treatment with propolis at 45 °C the reduction in weight dropped to zero since the mites died immediately, no change in weight. This phenomenon of total mite death was also observed at 40 °C for mites from adult workers where no change in weight was observed after treatment. The lack of weight change after treatment of mites from adult workers with 4% propolis at 40 °C agrees with the absence of heat production indicating that the mites were dead.

Table 1
The effect of treatment of *V. destructor* mites, from drone brood, with 4% propolis in 55% ethanol on the Q_{10} values[a]

Temperature shift in K	Before treatment		After treatment	
	$Q_{10\,heat}$	$Q_{10\,oxygen}$	$Q_{10\,heat}$	$Q_{10\,oxygen}$
25–35	2.4	2.3	2.0	1.5
30–40	1.1	1.2	1.7	1.4

[a] $Q_{10\,heat}$ and $Q_{10\,oxygen}$ represent the change in heat production and oxygen consumption rates due to change of temperature by 10 K. Twenty to 30 and 50–60 mites per calorimetric and respirometric experiments, respectively.

The typical power–time curves of *Varroa* mites are structured at the normal hive temperatures due to mite locomotor activity. But at elevated temperatures the curve is highly smoothed on a higher level, although it lasts for a short period of time (Fig. 5). Treatment of *Varroa* mites with propolis makes the curve lose its structure and the latter becomes nearly smooth. Though the specific heat production rate at 45 °C was higher than that at 35 °C, treatment of the mites with propolis has a stronger impact on the heat production rate at 45 °C reducing the mean value by 99.8 and

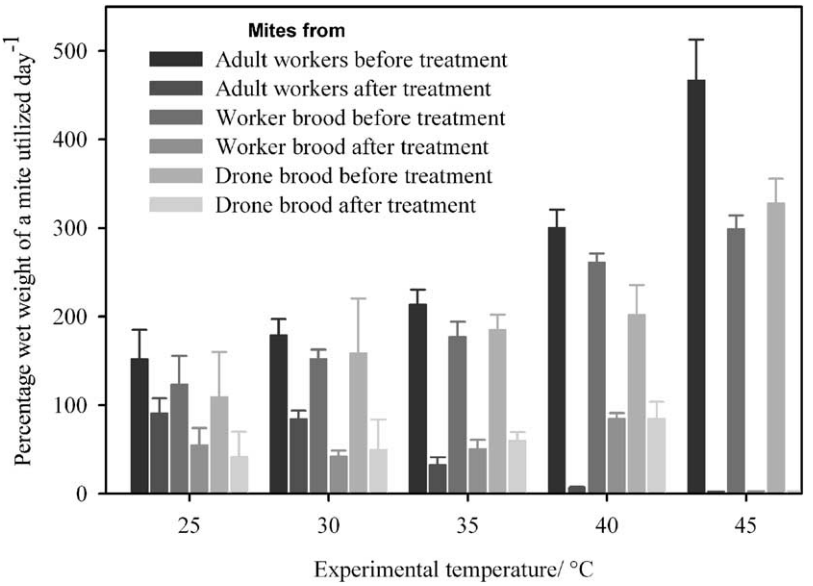

Fig. 4. Experimentally determined weight loss of the mite *V. destructor*, extrapolated to a hypothetical value per day and presented as the percentage of the initial wet weight, under starvation and different experimental temperature conditions before and after treatment with 4% propolis. $n = 5$, 25–30 mites per experiment. Treatment with 55% ethanol (control) reduced the weight loss rate by 7–12% regardless of mites' origin and temperature.

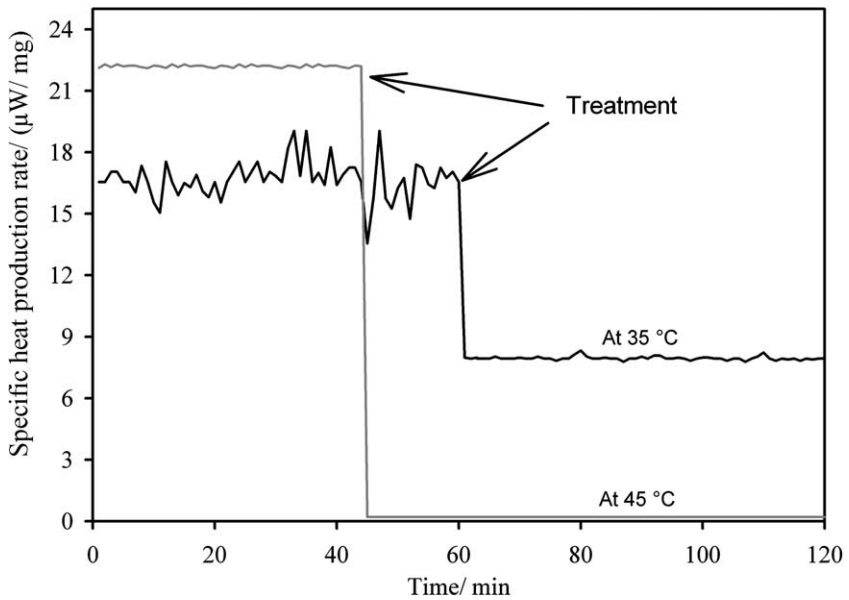

Fig. 5. Effect of treatment of *V. destructor* mites with 4% propolis on the structure and level of the $p - t$ curve in a typical calorimetric experiment with 30 mites from adult workers at 35 and 45 °C. A time gap of 30 min (omitted in the graph) was required after treatment for the thermal equilibration of the calorimeter.

70%, respectively, and smoothing the curve in both cases.

4. Discussion

The higher heat production rate of the phoretic mites (mites from adult workers) at lower temperatures could be an indication that they are adapted to the low temperatures they are confronted with on the workers' surface during the flying activity. It is obvious that the phoretic mites are often exposed to such lower temperatures than mites on worker broods since the temperature of the beehive is highly regulated, but not the surface of a flying bee. The heat production rate of *Varroa* mites from adult workers, worker brood and drone brood grew with increasing temperature indicating their thermo-conformer physiological nature, and remains at a nearly constant level between 30 and 40 °C, with a slight increase between 30 and 35 °C, demonstrating that this range is their normal physiological and/or tolerable temperature range. It was demonstrated [38] that *Varroa* mites prefer temperatures of 34 °C and below. This shows that the higher temperature range of 35–40 °C marked by a similar heat production rate as at the preferred, lower temperature range, is tolerable by *Varroa* mites, though not preferred. In addition to that, there is no significant difference in the heat production rate of mites from the three groups at a particular temperature in this normal physiological temperature range indicating that they are equally well adapted, except the mites from adult workers with a significantly higher heat production rate at 35 °C.

The very similar heat production rate of the mites from drone brood and worker brood in the temperature range of 30–40 °C contradicts the notion [39] that *Varroa* mites reproduce better in drone brood than in worker brood due to the convenient and slightly lower temperature in the former. Our suggestion is that the reasons for the higher reproduction rate of *V. destructor* in drone brood could be due to other factors, like the prolonged capped developmental stage of the drone brood. At lower temperatures, however, the mites from drone brood show a slightly higher heat production rate than those from worker brood. This is easily explained by the fact that the worker brood is usually located at the centre of the comb, with a relatively higher temperature [39], and the drone brood more to the periphery. The very high heat production rate at 45 °C lasted for 90–180 min, followed by a sharp decline of the curve, displaying that this temperature is extreme for the mites and they tried to escape, leading to their restlessness and very high metabolic rates. Several authors used high temperatures to kill mites trapped in worker- and drone broods without or with very little damage to the brood [28,32,40] and also from the surface of adult workers [41]. A combined treatment with heat and bee repellent (used to avoid aggregation of bees) produced a strong synergistic varroacidal action [41].

Mites from worker- and drone broods displayed similar responses to 4% propolis, but phoretic mites showed a different response, indicating different behaviour and physiological condition. The death of phoretic mites after treatment with 4% propolis at 40 °C demonstrates that these mites are highly vulnerable to treatment even at this temperature whereas the mites from the worker- and drone broods survived with a reduction in their heat production rates by only 68 and 60%, respectively. Treatment of mites at 45 °C resulted in an immediate death displaying the synergistic effect of propolis and temperature.

In addition to displaying the effect of temperature and propolis on the metabolic rate of *V. destructor*, the oxygen consumption rates from Fig. 3 could help us to find out how long we can run the calorimetric experiments in the closed calorimetric vessels without the need for ventilation. If we consider a mean oxygen consumption rate of 2.0 $\mu l\,mg^{-1}\,h^{-1}$ the total oxygen consumed within 5 h (the maximum experimental time used) will be 10 $\mu l\,mg^{-1}$. Considering 30 mites (ca. 12 mg), the total amount of oxygen consumed during the experimental period is 120 μl. The calorimetric vessel has a volume of 12,000 μl. Since oxygen makes up 21% of atmospheric gas, the total amount of oxygen in the vessel is 2520 μl. During the experimental period the partial pressure of oxygen in the gas volume would be reduced from the original 21–20%. This shows that we can run the calorimetric experiments without any problem of oxygen deficiency.

The treatment of mites with propolis reduced heat production and oxygen consumption rates proportionally indicating that the treatment affects both of them, which are directly related to each other only in case of

aerobic respiration. The similar values of both curves show that the heat produced during metabolism is due to aerobic respiration. This means that one may use the manometeric method in the metabolic investigation of *Varroa* mites instead of the expensive calorimeters. The higher Q_{10} value for the temperature increase from 25 to 35 °C, as compared to that of 30–40 °C, before treatment clearly indicates that the metabolism of the mites is well adapted to ambient temperatures around 35 °C which is the temperature found in a beehive. The after treatment Q_{10} values decreased for both the heat production and oxygen consumption rates for the temperature shift of 25–35 °C, as compared to the values before treatment indicating that the treatment has weakened the mites. But in case of the temperature change of 30–40 °C the Q_{10} values after treatment were paradoxically higher than before treatment. The most probable explanation for this could be that the mites from drone brood are agitated due to propolis treatment at 40 °C and trying to escape or that the higher metabolic rates are indications of metabolic inefficiency, introduced due to propolis poisoning.

It becomes clear from the heat production rate and utilization of reserve food during starvation that mites from adult workers behave differently than those from the brood stages. The phoretic mites have relatively higher heat production and resource utilization rates at almost all experimental temperatures. This can be due to two reasons: (i) since they are fully grown up they may posses a larger proportion of actively metabolising muscles than reserve food in their body compared to the mites from the brood stage, contributing to a higher metabolic rate and/or (ii) as an adaptation to their way of life, actively attaching themselves to flying bees not to fall down, that needs a larger amount of energy, the phoretic mites might have developed efficient metabolising system.

Acknowledgements

We would like to thank Dr. Benedict Polaczek for his help in the collection of mites. We express our gratefulness to the Holeta Bee Research Centre (Ethiopia) and Dr. Eyualem Abebe, for providing us the propolis sample. Thanks also go to Dr. Klaus Wallner for performing the acaricidal residue analysis of the propolis sample. Last but not least we would like to thank the DAAD (Deutscher Akademischer Austauschdienst) for the financial support of A.G.

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