4.0 Microcalorimetric and Respirometric Investigations of the Effect of Temperature on the Antivarroa Action of Propolis

4.1 Abstract

The antivarroa action of propolis and its synergism with temperature was investigated calorimetrically and respirometerically using female *Varroa destructor* (Anderson and Trueman) mites from adult workers, worker and drone brood.

The treatment of Varroa mites with 4% propolis in ethanol affected the metabolic activity of the mites with the effect directly related to the temperature of treatment. The mites collected from worker and drone brood reacted similarly to propolis treatment at different temperatures. In contrast to that, 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 brood by 68% and 60%, respectively. 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.

Exposure of mites to 45 °C agitated them, as witnessed by the elevated heat production rates, 23.5 ± 2.5 μW mg^{-1} compared to 14.4 ±1.0 μW mg^{-1} at 35 °C. 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 than the prolonged use of either method.

4.2 Introduction

Varroa destructor (Anderson and Trueman) is a serious ectoparasitic parasite of the western honeybee *Apis mellifera* L., infesting both feral and managed colonies. It has caused the destruction of numerous colonies. This led to the reduction in the number of beekeepers and the harvest of honey and other bee products in different parts of the world, in the last three decades (Peng et al. 1987, Delfinado-Baker 1988, Kovac and Crailsheim 1988, Matheson 1994, Kraus and Page 1995, De Jong 1997, Finley et al. 1997). To save their colonies from obliteration beekeepers are using acaricides as short-term solutions. The use of chemical acaricides is not, however, free from drawbacks: accumulation of residues in bee products (Kubik et al. 1995, Wallner 1995, Stürz and Wallner 1997, Bogdanov et al. 1998, Wallner 1999), hazards to the bees and/or the beekeeper (Ellis Jr. 2001), and the emergence of acaricide resistant mites (Milani 1994, Lodesani et al. 1995)

There is an ever increasing number of reports concerning the resistance of *Varroa destructor* against various acaricides in different parts of the world. The 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 (Rosenkranz 1987, Maul et al. 1988, Fries and Hansen 1993, Engels 1994, Calis et al. 1998), heat treatment of infested brood using a Mitezapper (Huang 2001) and infested adult workers (Tabor and Ambrose 2001), a combination of biotechnical control and acaricide treatment (Fries 1991, Calis et al. 1998), a combination of biotechnical control and heat treatment (Rosenkranz 1987, Engels 1994) 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 (Imdorf et al. 1999, Ellis Jr. 2001). 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 these groups of chemicals produced by plants is propolis.

A laboratory assay of the antivarroa actions of propolis in Chapter 3 displayed that it possesses both narcotic and lethal actions with 10% propolis killing 100% of Varroa mites, even with a very short contact time of 5 s. Lower concentrations display varying degrees of suppression of the metabolic rate of the mites.

In this chapter it will be demonstrated, by calorimetric and respirometric methods, if the varroacidal action of propolis can be augmented by a simultaneous exposure of the mites to higher or lower temperature extremes.

4.3 Materials and Methods

4.3.1 Animal material

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

Female *Varroa destructor* mites were collected from adult workers, worker brood, and drone brood. The collection of mites from the brood stage was carried out at room temperature by opening and inspecting healthy brood. During the collection process mites were kept in a Petridish on the corresponding bee pupae in order to avoid starvation. Newly moulted adult mites

were excluded from the experiment since they might be possible sources of error given that the 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.

4.3.2 Calorimetric experiments

The calorimetric experiments were performed using two isoperibolic calorimeters: (i) a Biocalorimeter B.C.P. (Messgeräte Vertrieb, München, Germany) with a sensitivity of 44.73 μV mW^{-1} and a vessel volume of 12 cm^3 and (ii) a THERMANALYSE calorimeter (Messgeräte Vertrieb, München, Germany) with a sensitivity of 40.65 μV mW^{-1} 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 conducted at temperatures of 25, 30, 35, 40, 45, and 50 °C with mites from adult workers, worker brood, and drone brood. 20 to 30 mites were weighed before each experiment using an analytical balance (Sauter, Ebingen, Germany) of 0.1 mg sensitivity, transferred into the calorimeter, and the heat production rate was recorded for 2 to 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 to 180 min after exposure. Recording of heat production rate was stopped after the pre-selected experimental time and mites were removed from the calorimeter, and weighed again immediately to find out the change in weight. The rate of utilization of reserve food under starving conditions and, thus, the change of weight help to illustrate the amount of hemolymph 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 (4.3.3). Having blotted the excess fluid from their surface the mites were weighed again to obtain the after-treatment initial weight, and they were put back into the calorimeter and their heat production rate was recorded further for 3 to 5 h. The mites were weighed at the end of the calorimetric experiment to determine the rate of weight change. The hypothetical weight loss of a mite per day was extrapolated from the weight loss during the experimental period. In this regard the rate of weight change was presented as wet weight loss per mite per day before and after treatment with 4% propolis at the different experimental temperatures.

4.3.3 Treatment of mites with propolis

Since the goal of these experiments was to observe the effect of temperature on the antivarroa action of a sublethal dose of propolis, a 4% propolis in 55% ethanol was used. It made no sense to apply a lethal dose of propolis because 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 placed in a clean Petridish, on top of a 3 cm x 3 cm tissue paper (Kimwipes™ Lite 200, Kimberly – Clark™). Mites were treated for 30 s after applying 250 µL of the 4% propolis solution on the tissue paper, not directly on the mites. After the allocated time the treatment was ended by removing the mites from the Petridish, 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 the conclusion of the treatment was important, since it otherwise would have interfered with the calorimetric signal due to evaporational heat loss. The treated and blotted mites were weighed again, placed back into the calorimetric vessel, and their heat production rate was recorded. Control experiments for each experimental group were carried out by treating the mites with 55% ethanol as well as distilled water.

4.3.4 Respirometric experiments

The effect of temperature and propolis treatment on the oxygen consumption rate of *Varroa destructor* mites from drone brood was investigated at 25, 30, 35, 40, 45, and 50 °C using manometric methods. The respiration experiments were conducted using Warburg vessels of about 12 cm^3 volume and 50 to 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 (1mm thick) of porous spongy material with a pore size of ca. 0.8 mm, which allows air to enter but not the mites. Recording the oxygen consumption rate was started after a temperature equilibration time of 30 min, and further recording was made in intervals of 30 min, for 2 h before treatment. The respirometric experimental time for each temperature set-up was equal to the calorimetric experimental times. Each measurement was conducted 5 times, but 9 times in case of measurements with 45 °C since the experimental time of the latter temperature set-up was short compared to that of the others 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 various developmental stages will be made to see if the mites have different responses.

4.3.5 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 were determined using either the student's t-test, or paired sample t-test, or 1-way ANOVA and Tukey's HSD post hoc test with a critical value of $\alpha = 0.05$.

4.4 Results

The mites obtained from adult 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 brood showed 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 (students t-test, $p = 0.03$). At an experimental temperature of 25 °C, the mites collected from adult workers displayed a significantly higher specific heat production rate of 8.4 ± 1.4 μW mg^{-1}, compared to 5.0 ± 0.4 μW mg^{-1} ($p = 0.025$) and 6.1 ± 1.2 μW mg^{-1} ($p = 0.033$), for mites from worker brood and drone brood, respectively (1-way ANOVA, $p = 0.019$, and Tukey's HSD post hoc test) (Fig. 4.1). There was no significant difference ($p = 0.16$) between the heat production rates of mites from worker and drone brood. The mites from adult workers also had a significantly higher specific heat production rate of 17.4 ± 1.1 μW mg^{-1} at 35 °C, compared to those from worker brood with 14.3 ± 0.9 μW mg^{-1} ($p = 0.041$) and drone brood with 14.6 ± 1.0 μW mg^{-1} ($p = 0.036$) (1-way ANOVA, $p = 0.025$, and Tukey's HSD post hoc test). Mites from worker and drone brood showed no significantly different heat production rates ($p = 0.35$) at 35 °C too. There was no significant differences in the heat production rates of the different mites at 30 °C and 40 °C ($p = 0.08$, and $p = 0.12$, respectively, 1-way ANOVA). Regardless of where the mites were obtained from, the heat production rates increased with raising calorimetric temperature, achieving constant rates between 35 °C and 40 °C. With the shift of temperature from 40 to 45 °C the heat production rate increased drastically from 14.6 to 23.5 μW mg^{-1}, in case of mites from worker brood, as an example. The high heat production rates at this elevated temperature (45 °C) lasted

for a short period of time, only 90 to 180 min. After this time interval the *p-t* curves declined, due to the death of mites; death ensued faster in case of mites from adult workers than those from worker and drone brood.

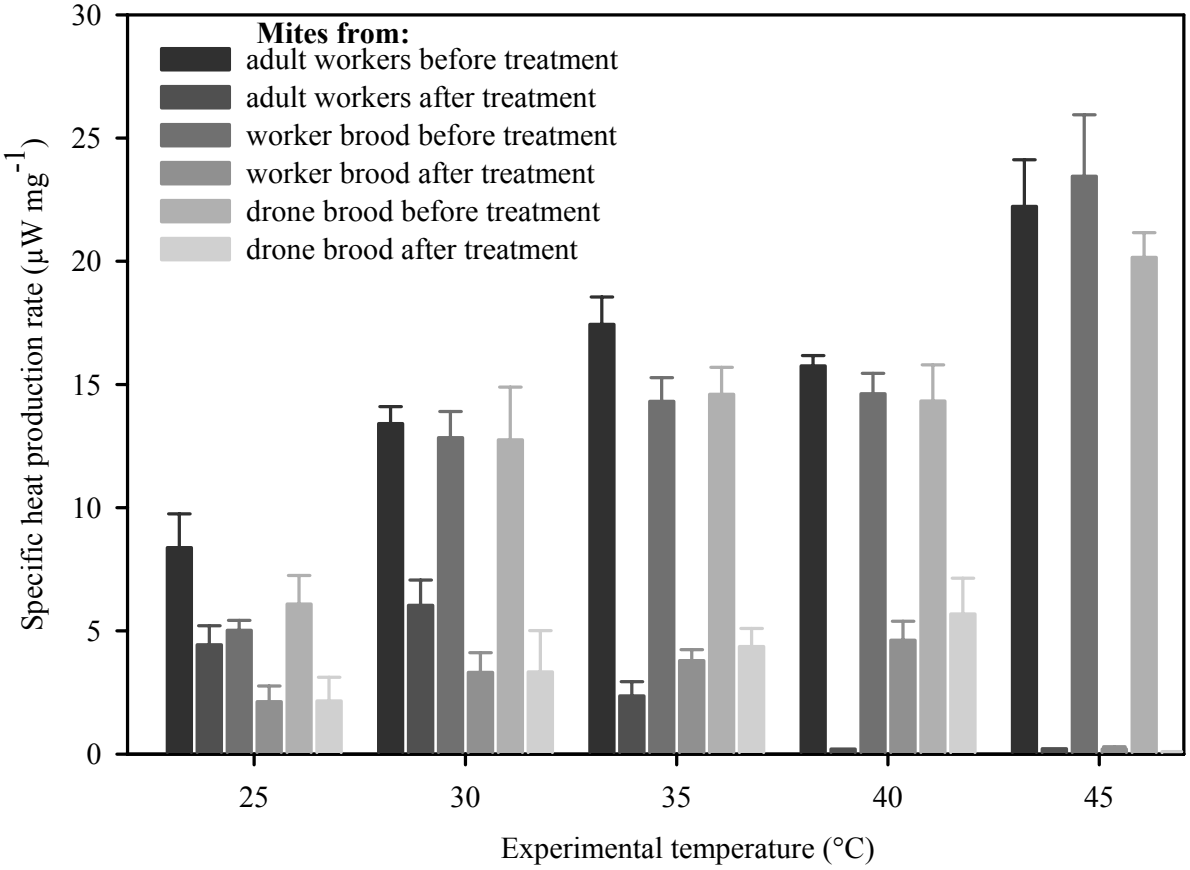

Fig. 4.1 The effect of temperature on the specific heat production rate of *Varroa destructor* mites before and after treatment with 4% propolis. Twenty to 30 mites per experiment, n = 5, mean ± s.d. The treatment with 55% ethanol (control) reduced the heat production rate by 5 % to 9% regardless of temperature and origin of mites.

Treatment of mites with 4% propolis resulted in a reduction of the heat production rate. The extent of reduction increased with the experimental temperature, especially in case of mites obtained from adult workers (Fig. 4.2). Mites from adult workers were all dead after treatment with 4% propolis at 40 °C, whereas those from worker and drone brood showed a reduction in the heat production rate by 68% and 60%, respectively. A mortality of 100% was achieved after treatment with 4% propolis at 45 °C, regardless of the origin of mites, the heat production rate dropping to the base line (Fig. 4.1 and 4.2). Varroa mites from worker and drone brood showed nearly similar responses to the treatment with propolis at different experimental temperatures, whereas mites from adult workers had a different response (Fig. 4.2). The control experiments, treatments with 55% ethanol rendered a reduction of the specific heat production rate by 8% to 11% regardless of the temperature of treatment.

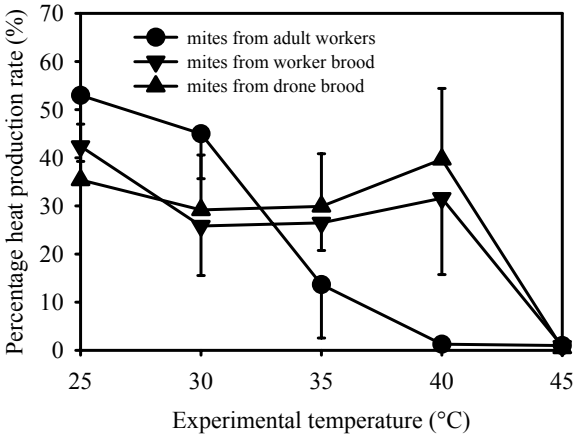

Fig. 4.2 Effect of temperature on the percentage residual heat production rate (p after treatment / p before treatment x 100) of *Varroa destructor* mites after treatment with 4% propolis. Twenty to 30 mites per experiment, n = 5, mean ± s.d. After treatment with 55% ethanol (control) the residual heat production rate lay between 91 % and 95% regardless of the experimental temperature and origin of mites.

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. 4.3). 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 increased 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 only 1.1, and the oxygen consumption rate grew by a factor of 1.2. After treatment with 4% propolis the changes in the rates of heat production and oxygen consumption showed different patterns 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 shift from 30 to 40 °C amounted to 1.7, and the oxygen consumption changed by a factor of 1.5 (Table 4.1). The treatment with 55% ethanol (control experiment) reduced the oxygen consumption rate by 6 to 10% independent of the experimental temperature. The other control experiments showed no effect.

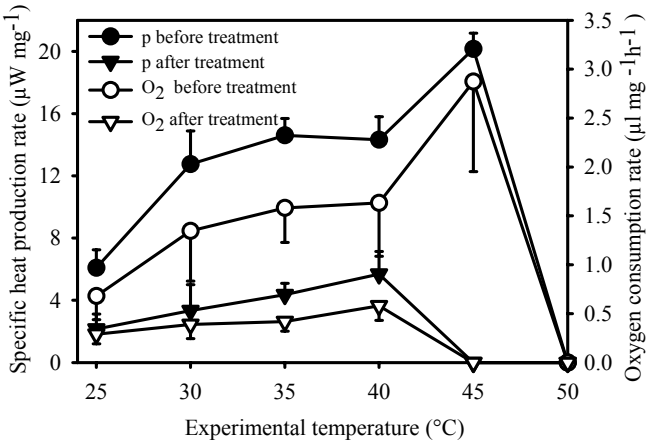

Fig. 4.3 Effect of temperature on the specific heat production rate (p) and oxygen (O_2) consumption rate of *Varroa destructor* mites from drone brood before and after treatment with 4% propolis. 20 to 30, and 50 to 60 mites per experiment, for the calorimetric and respirometric experiments, respectively. n = 5 (but n = 9 for the respirometric measurements at 45°C), mean ± s.d. The oxygen consumption rate is reduced by 5% to 11% and the heat production rate by 5% to 9% in the control group

Utilization of own reserve food by Varroa mites, displayed by the loss of wet weight during starvation, was highly affected by the experimental temperature. The mites collected from adult workers lost a higher proportion of their body weight per starvation hour than those from drone and worker brood. A mite from an adult worker may utilize 1.5 fold of its own weight per day at 25 °C, whereas those from worker and drone brood could utilize 1.2 and 1.1 fold of their own weight, respectively, at this temperature. The rate of wet weight loss by mites from adult workers was significantly different from the other two, which do not display significant difference among each other (1-way ANOVA, Tukey's HSD post hoc test $\alpha = 0.05$).

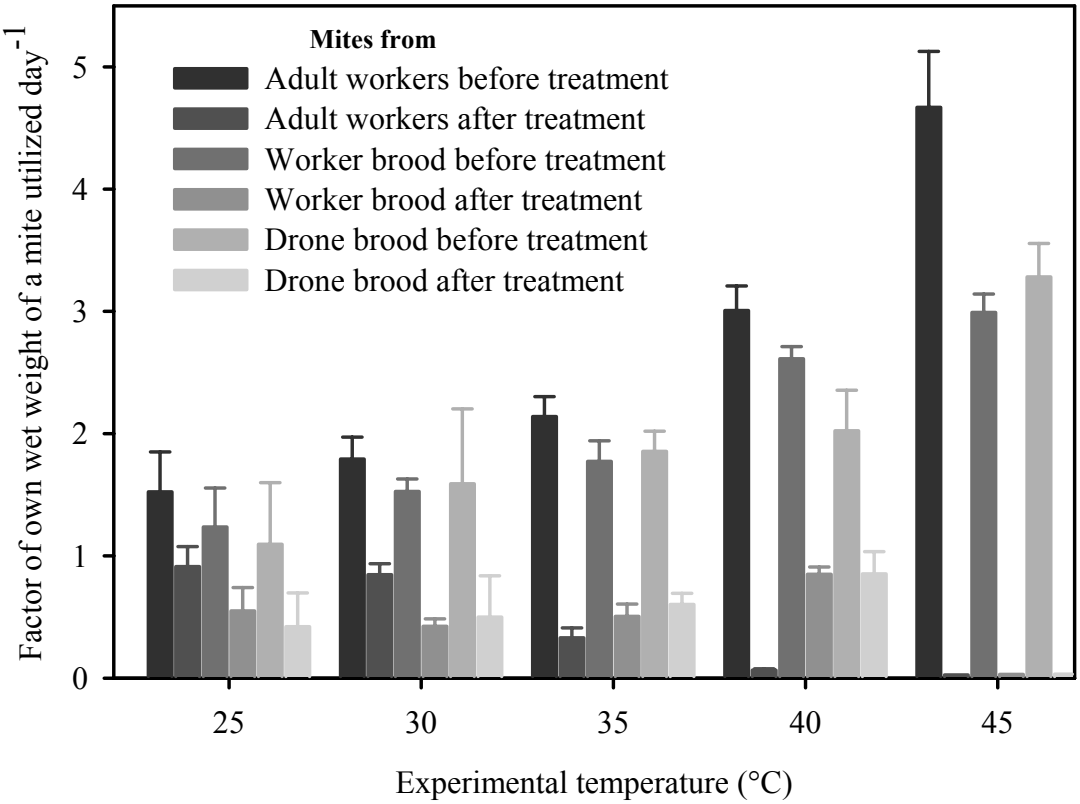

Fig. 4.4 Experimentally determined weight loss of the mite *Varroa destructor*, extrapolated to a hypothetical value per day and presented as a factor of the initial wet weight, under starvation and different experimental temperature conditions, before and after treatment with 4% propolis. n = 5, 25 to 30 mites per experiment. Treatment with 55% ethanol (control) reduced the weight loss rate by 7 % to 12% regardless of mites' origin and temperature.

At 45 °C mites from adult workers, worker brood or drone brood could theoretically utilize 4.7, 3.0 or 3.3 fold, respectively, of their own weight per day (Fig. 4.4). Percentage of wet weight reduction 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, and, therefore, there was 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 is in agreement with the absence of heat production, indicating that the mites were dead.

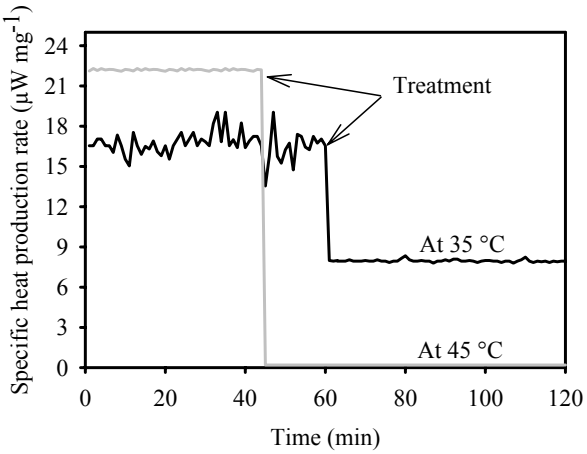

Fig. 4.5 Effect of treatment of *Varroa destructor* mites with 4% propolis on the structure and level of the *p-t* curve, in a typical calorimetric experiment with 30 mites from adult workers at 35 °C and 45°C. A time gap of 30 minutes (omitted in the graph) was required after treatment for the thermal equilibration of the calorimeter.

A typical power-time curve of Varroa mites is usually structured at the normal hive temperature due to mite locomotor activities. But at elevated temperatures the curves are highly smoothed and lie at higher levels, although they last for a short period of time (Fig. 4.5). Treatment of mites with propolis made the *p-t* curves lose their structures and the latter became nearly smooth. Though the specific heat production rate at 45 °C was higher than that at 35 °C, treatment of mites with propolis had a stronger impact on the former, reducing the mean heat production rates by 99.8% and 70%, respectively. In addition to that both curves after treatment were highly smoothed.

Table 4.1 The effect of propolis on Q_{10}
The effect of treatment of *Varroa destructor* mites with 4% Propolis in 55% ethanol on the Q_{10} values. $Q_{10\ Heat}$ and $Q_{10\ Oxygen}$ represent the change in heat production and oxygen consumption rates due to change of temperature by 10 K. 20 to 30 and 50 to 60 mites per calorimetric and respirometric experiments, respectively.

Temperature shift	Before treatment		After treatment	
	$Q_{10\ Heat}$	$Q_{10\ Oxygen}$	$Q_{10\ Heat}$	$Q_{10\ Oxygen}$
25 to 35 °C	2.4	2.3	2.0	1.5
30 to 40 °C	1.1	1.2	1.7	1.4

4.5 Discussion

The higher heat production rate of the phoretic mites at lower temperatures could be an indication that they are adapted to the low temperatures which they are confronted with on the workers' surface during the flying activity of the latter. It is obvious that the phoretic mites are

more often exposed to such lower temperatures than mites on brood in capped cells, since the temperature of the beehive is highly regulated, whereas the surface of a flying bee is not. 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; the rate remained at a nearly constant level between 30 and 40 °C, with a slight increase between 30 and 35 °C. The constant level of heat production rate between 30 and 40 °C demonstrates that this range is their normal physiological and/or tolerable temperature range. It was demonstrated by Rosenkranz (1985) that Varroa mites prefer temperatures of 34 °C and below. This shows that the higher temperature range of 35 to 40 °C, marked by a similar heat production rate as at the preferred lower temperature range, is tolerated by Varroa mites, though it is not preferred. In addition to this, there is no significant difference in the heat production rate of the mites from the three groups at a particular temperature in this normal physiological temperature range, indicating that they are all equally well adapted. The mites from adult workers, however, had a significantly higher heat production rate at 35 °C.

The very similar heat production rate of mites from drone brood and worker brood in the temperature range of 30 to 40 °C contradicts the idea by Kraus et al. (1998) that Varroa mites reproduce better in drone brood than in worker brood due to the convenient and slightly lower temperature in the former. It is suggested here that the reasons for the higher reproduction rate of *Varroa destructor* in drone brood could be due to other factors, such as the prolonged capped developmental stage of the drone brood. At lower temperatures, however, the mites from drone brood showed 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 (Kraus et al. 1998), and that the drone brood is located more to the periphery. The very high heat production rate at 45 °C lasted for 90 to 180 min, followed by a sharp decline of the curve, displaying that this temperature is extreme for the mites, and that they were trying to escape, leading to their restlessness and very high metabolic rates. Several researchers used high temperatures to kill mites trapped in worker and drone brood without or with very little damage to the brood (Rosenkranz 1987, Brødsgaard and Hansen 1994, Huang 2001), and also from the surface of adult workers (Hoppe and Ritter 1987). A combined treatment of heat and bee repellent (used to avoid aggregation of bees) produced a strong synergistic varroacidal action (Hoppe and Ritter 1987).

Mites from worker and drone brood displayed similar responses to 4% propolis, but phoretic mites showed a different response, indicating their altered behaviour and/or physiological conditions. The death of phoretic mites after treatment with 4% propolis at 40 °C

demonstrates that these mites are highly vulnerable to treatment at this temperature, whereas the mites from worker and drone brood 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 *Varroa destructor*, the oxygen consumption rates displayed in Fig. 4.3 gives evidence about the length of time one can run the calorimetric experiments with mites, without the need for ventilation. If we consider a mean oxygen consumption rate of 2.0 µl mg^{-1} h^{-1}, the total oxygen consumed within 5 h (the maximum experimental time used) will be 10 µl 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 calorimetric vessel would be reduced from the original 21% to 20%. This shows that the calorimetric experiments, even by using a closed calorimetric vessel, can be run without any problem of oxygen deficiency at least for the first five hours. In addition to this, since the calorimetric vessel is not sealed oxygen can not be a limiting factor.

The treatment of mites with propolis reduced the 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 to 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 with the shift of temperature from 25 to 35 °C, as compared to the values before treatment, indicating that the treatment has weakened the mites. In the case of change of temperature from 30 to 40 °C, however, 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 were agitated due to propolis treatment at 40 °C and that they were 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, contributing to a higher metabolic rate as compared to the mites from the brood stage, which possess a larger proportion of reserve food, and/or (ii) as an adaptation to their way of life i.e. actively attaching themselves to flying bees, in order not to fall down, which needs a larger amount of energy, the phoretic mites might have developed efficient metabolic system.