5.0 Comparative Investigations of the Antivarroa Actions of Propolis from Different Geographic Origins

5.1 Abstract

Varroosis of the western honeybee, *Apis mellifera* L. has become a serious problem for the beekeeping industry in the last 3 decades. Caused by the parasitic mite *Varroa destructor* (Anderson and Trueman), varroosis destroys colonies within 2 to 5 years, unless treated. Due to the emergence of mites resistant to the currently existing acaricides, and the undesirable residue problems caused by the latter, the search for new acaricides is becoming increasingly important. Propolis has already displayed Varroa narcotizing and varroacidal effects in chapters 3 and 4. In this chapter the antivarroa actions of propolis from different geographic origins are investigate *in vitro*, using Petridish bioassay and calorimetric methods.

All propolis samples were extracted in 70% ethanol, and used in 55% ethanol for treatment. In addition to this, 1 sample was extracted, and further used in water. Treatment of mites was carried out by placing them on top of a paper towel in a Petridish, wetted with propolis solution, and keeping them in contact for 30 s.

Treatment with propolis solution resulted in narcosis and the eventual death of mites, regardless of the geographic origin of propolis. The strength of narcosis, illustrated by the time needed for potentially recoverable mites to recover, varied from sample to sample. The lethal effects displayed by a certain concentration of the various propolis samples were not, however, significantly different from each other, regardless of the propolis origin. Sublethal doses of propolis caused drops in the heat production rate and smoothing of the power-time (*p*-*t*) curves.

The different concentrations of water-extracted propolis showed significantly lower antivarroa actions, demonstrated by the weaker narcotic effects, less mortality of mites, and lower reduction of heat production rates compared to ethanol-extracted propolis samples.

5.2 Introduction

Varroa destructor (Anderson and Trueman) has become one of the most destructive parasites of the western honeybee *Apis mellifera* in the last 3 decades, causing death and withering of several colonies in different parts of the world (Boecking and Spivak 1999). In order to minimize mite infestation rate and subsequently reduce or prevent colony death, beekeepers are using different types of acaricides in the beehive environment.

The widespread and unwise use of synthetic acaricides by beekeepers to combat colony loss has led to the emergence of *Varroa destructor* mites resistant to most of the acaricides in use

today in different parts of the world. Mites resistant 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). In addition to the problem of resistance by mites, acaricidal use is also associated with the contamination of hive products (Kubik et al. 1995, Wallner 1995, Stürz and Wallner 1997, Bogdanov et al. 1998, Wallner 1999), rendering them inconvenient for human use. Honeybee products that suffer from acaricidal contamination are mainly wax and propolis, largely due to the hydrophobic nature of most acaricides.

In order to prevent colony death researchers are searching for acaricides free from the aforementioned problems and are trying to select Varroa-resistant bee races. Though the selection of resistant bee races is a long-lasting solution, it takes longer time. In the meantime, however, colonies have to be treated with acaricides to prevent their loss. In this aspect, the solution which is second to none is the search and use of acaricides free from residue problems. Most of the researches in the area of acaricide screening and search nowadays are concentrating mainly on the use of organic acids and natural products, such as essential oils, because they naturally occur in the beehive and are non-toxic to humans and bees (Kraus et al. 1994). One of these natural products that does not have the problems mentioned for synthetic acaricides, and naturally occurs in the beehive is propolis. Experimental results in the previous chapters displayed that propolis exhibits Varroa weakening and varroacidal actions.

Propolis is a resinous product of the honeybees accumulated in the beehive for different purposes, such as varnish, sealant, putty, bactericide and fungicide. Due to the variation in the types of source plants, propolis samples from various geographical origins have different chemical compositions. This variation may have an impact on the antivarroa actions of the samples. The aim of this investigation was, therefore, to compare the antivarroa actions of propolis of different geographic origins. For this purpose samples were obtained from completely different geographic environments. The detailed mechanisms of action of propolis on Varroa mites shall be investigated using calorimetric methods in addition to the standard Petridish bioassay methods.

5.3 Materials and Methods

5.3.1 Propolis sources

In order to compare the differences in the antivarroa activities of propolis from diverse geographical origins, several samples were obtained from different countries by personal contact

with beekeepers and bee researchers in the corresponding countries. The details of propolis sources including the honey bee species that collected them are listed in Table 5.1. Collection of all propolis samples, except the ones bought from local bazaars, according to the information obtained from the donors, was done by scrapping them from the inner wall of the hive and frames of the honey comb. The different propolis samples were acquired as solid raw samples.

Table 5.1 Description of the different propolis samples
Countries of origin, abbreviated names used in the text, colour, and collecting honeybee species of the different propolis samples used. Rus1 was bought from a Russian natural products shop on the "International Green week" exhibition and bazaar (2001) in Berlin. K1 was bought in a bazaar in Almati, Kazakhstan. The exact subspecies of the collecting bees are, therefore, not known. WEP is the water extracted solution of the sample G1.

Geographical Origin	Sample name	Colour	Source Honeybee
Bogota-Colombia	C1	Dark brown	*Tetragonsica angustula*
Holeta-Ethiopia	E1	Dark brown	*A. m. scutellata*
Berlin-Germany	G1	Golden brown	*A. m. carnica*
Berlin-Germany	WEP	Golden brown	*A. m. carnica*
Almati-Kazakhstan	K1	Dark brown	*A. mellifera?*
Russia	Rus1	Greenish brown	*A. mellifera?*
Lublienic-Poland	P1	Brown	*A.m. mellifera*
Opole-Poland	P2	Brown	*A.m. mellifera*
Gradkow-Poland	P3	Golden brown	*A.m. mellifera*
Nysa-Poland	P4	Brown	*A.m. mellifera*
Graham Town–S. Africa	SA1	Dark brown	*A. m. capensis*
Graham Town–S. Africa	SA3	Dark brown	*A. m. capensis*
Graham Town–S. Africa	SA3	Dark brown	*A. m. capensis*
Graham Town–S. Africa	SA5	Dark brown	*A. m. capensis*
Graham Town–S. Africa	SA6	Dark brown	*A. m. capensis*
Graham Town–S. Africa	SA8	Dark brown	*A. m. capensis*
Graham Town–S. Africa	SA11	Dark brown	*A. m. capensis*
Udine-Italy	I1	Brown	*A. m. ligustica*

5.3.2 Preparation of propolis extracts

All samples were extracted in 70% ethanol and used in 55% ethanol for the bioassays. As already described in Chapter 3, 55% ethanol was used as a solvent in the treatment solution to minimize/avoid the effect of strongly concentrated ethanol on the experimental organism. It would have been desirable to prepare different types of extracts from each propolis sample, and to compare their antivarroa activities. However, as the sample sizes of the acquired propolis were too small, it was not possible to do this. Extraction was, therefore, done only with 70% ethanol for all samples but one; a water-extracted propolis (WEP) solution was prepared for the sample from Germany (G1) by extracting it in distilled water, since it was available in the needed quantity. The method of extraction of propolis in water was the same as that used for the extraction in alcohol, with exception of the solvent. This extract was dissolved in distilled water for treatments. Stock solutions (10%) of both the WEP and EEP were prepared in water and 55%

ethanol, respectively, and the treatment solutions, i.e. 2%, 4%, 6%, and 8% were prepared by diluting the stock solutions in the corresponding solvents.

5.3.3 Biological material

The biological material used for this investigation was the honeybee parasitic mite *Varroa destructor* collected from drone brood. As the physiological status and weight of an egg laying and a phoretic female mite could differ, with the weight of the former increasing by up to 30% of that of the latter (Steiner et al. 1995), only mites at the late developmental stages of the drone brood (brown to dark skin pupal stages, which are older than 20 days of brood development) were used in the investigations. Infested drone brood combs around the 20^{th} day of brood development were obtained from the research beehive of the Institute of Zoology, Free University of Berlin. If the brood was not yet in the brown or dark skin developmental stage the comb was incubated further in an incubator at a temperature of 35 °C and RH of 65 ± 5% until the desired stage was achieved. Collection of mites was carried out by opening and inspecting individual brood cells. Any mite obtained from unhealthy (infected) brood was rejected since the physiological status of such mites could be different from those obtained from the healthy (non-infected) ones. During the collection process mites were put in a Petridish containing drone pupae in order to avoid starvation.

5.3.4 Petridish bioassay

The Petridish bioassay was done in order to compare the narcotic effects, and subsequently the lethality of various concentrations of the different propolis samples. Before treatment the mites were put in a Petridish on top of a 3 cm x 3 cm tissue paper (Kimwipes™ Lite 200, Kimberly–Clark™), in order to make handling of the mites easier. Treatment of the mites was done by applying 250 µl propolis solution of the desired propolis type and concentration on top of the tissue paper, not directly on the mites, and keeping them in contact for 30 s. The treatment was ended after the allocated time by removing the tissue paper containing the mites from the Petridish and placing it on a pad of paper towel. The mites were then immediately removed from the treatment tissue paper and placed on a clean paper towel for 1 min to blot the excess propolis solution on their surface. Blotting of the excess fluid from the mites' surface after conclusion of the treatment was especially important in the calorimetric experiments because it otherwise would interfere with the calorimetric signal due to evaporational heat loss of the excess fluid on the mites.

At the end of the treatment the activity of mites was observed by counting the number of narcotized mites at an interval of 30 min for 4 h, starting from the time at which the mites were removed from the paper towel, and placed in a Petridish for further observation. Activity of the mites was examined by gently prodding them with a blunt needle under a binocular microscope. Regardless of the nature of movement, whether only limbs or the whole body, a mite was considered as "active" if it showed even a slight movement, and "narcotized" if there was no movement of any body part. In between the observation times the treated mites were kept in a Petridish containing drone pupae (2 drone pupae per treatment i.e. 6 mites), and placed in an incubator at a temperature of 35 °C and an r.h. of 65 ± 5%. Each experiment was done with 6 mites and repeated 5x.

5.3.5 Calorimetric assay

A 4% w/v propolis solution was selected and used for all calorimetric experiments, since this concentration causes death in some mites and narcotizes and weakens the rest. The calorimeter used was a Biocalorimeter B.C.P.-600 (Messgeräte Vertrieb, München, Germany) with a sensitivity of 44.73 μV mW^{-1} and vessels with volume of 12 cm^3. Each calorimetric experiment was run by using 25 to 30 mites, and was repeated 3 times. The calorimetric signal was recorded using a one-channel recorder with a 1000x built-in amplifier (Kipp and Zonen, The Netherlands).

The heat production rate of mites before treatment was measured for 2 h. Recording was then stopped and the mites were removed from the calorimeter. They were then treated with 250 μl of 4% propolis by keeping them in contact for 30 s, as in the case of treatment with the Petridish bioassay experiment. After ending the treatment the mites were removed from the Petridish and then blotted by putting them on a clean paper towel for 5 min so that no fluid was left on their surface. They were then put back into the calorimetric vessel and the heat production rate was recorded for 2 to 4 h. At the end of the calorimetric experiment the number of dead mites was counted and noted, since it is important to differentiate between the total reduction of heat production rate (due to mite weakening and death), and the reduction of heat production rate of the survivor mites, due to weakening by the treatment.

In order to find out the total heat produced, and subsequently the heat production rate per unit time before and after treatment, the areas below the calorimetric *p-t* curves were determined using a planimeter (Digikon DK 4261, Kontron Registriertechnik GmbH, München, Germany). The *p-t* curves were also digitalized and transferred to a PC directly connected to the planimeter. The ASCII data was then imported to statistic and graphic PC programmes for further

computation and processing. The results of the calorimetric experiments were presented as specific heat production rates (p, μW mg^{-1}).

5.3.6 Scanning electron microscopy of propolis-treated Varroa mites

In order to evaluate the effect of propolis treatment on the morphology and surface structures female mites were treated with 10% EEP of G1 for 30 s. The surface of the treated mites was then scanned for any deformation or attack by propolis using a scanning electron microscope (FEI, Quanta 200) with a high tension of 10 kV.

5.3.7 Statistical analysis

The statistical tests used for the comparison of the results of different types of treatments were the repeated measures ANOVA of the general linear model (GLM), one-way ANOVA, Tukey's HSD post hoc test, paired sample t-test, and the harmonic mean, according to the nature of the data. Details of matrix construction and analysis method for each statistical test shall be briefly described in the results section up on using the test.

5.4 Results

Treatments with the different types and concentrations of propolis resulted in the narcosis of mites, immediately after treatment, and the activity of mites changed with observation time (Fig. 5.1 a to e). The measurement of mite activity involved repeated observations of the treated mites in time intervals of 30 min, to observe the change in their activity with the length of incubation time after the treatment. The control, a treatment with 55% ethanol, displayed that 90 to 100% of the mites were narcotized immediately after treatment and that narcosis lasted less than 5 min. Since there was no change in the activity of the control treatment mites after 5 min, with 100% of them being active, it was not necessary to display it as a graph with the other treatments, hence, it is missing in Fig. 5.1.

Treatments with 2%, 4%, and 6% WEP had no effect on the activity of mites even immediately after treatment, with all mites remaining active. This was unlike the corresponding weak concentrations of EEP which caused narcosis that changed with observation time. Since there was no activity change of the mites treated with the weak concentrations of WEP mentioned, these concentrations were not displayed in Fig. 5.1 a, b, and c. Though weak compared to the corresponding concentrations of EEP, 8% and 10% WEP showed narcotic and lethal effects that changed with observation time. Treatments with 8% and 10% WEP narcotized 40% and 50% of the mites, respectively, immediately after treatment and these values dropped to

16.7% and 36.7%, respectively, within 30 min. For the treatment with 10% WEP, the narcotic effect on some mites lasted longer and they became active in the time interval of 30 to 60 min, whereas in the case of 8% WEP no activity change was observed after the first 30 min. At the end of the experiment only 16.7% and 27.3% of the mites treated with 8% and 10% WEP, respectively, were dead.

Unlike the treatments with WEP and the control, treatments with different types and concentrations of EEP resulted in narcosis that lasted longer, the length of narcosis being dependent on the concentration of propolis. As it can be seen from Fig. 5.1 a to e, an increasing concentration of propolis resulted in a decreasing number of mites that recovered from narcosis with incubation time (Fig. 1 a versus c, d, and e). Though observation of mite activity was carried out for 4 continuous hours in intervals of 30 min, only the activity results for the first 1 hour and the last observations were presented in the figures. This is because no or very little activity change was observed during the other time intervals. If there was any activity change of mites taking place after the first 60 min observation time, it took place only in the second 60 min interval and remained constant thereafter. With decreasing concentration of propolis the percentage of mites that recovered from narcosis increased

In order to analyse the effect of (i) the type of propolis, (ii) propolis concentration, and (iii) the length of observation time on the activity of mites after treatment, a statistical test called repeated measures ANOVA of the General Linear Model (GLM) was used. This statistical test was run on a matrix containing the type of propolis (17 levels) and concentration of propolis (5 levels) as between subject factors, and the activity status of the mites at 30 min interval observation times (9 repeated observations - levels) as within subject factors. The test was run using a Statistic Programme (SPSS 11.0.1 for Windows), at a significance level of $\alpha = 0.05$. In the case of presence of significant differences within the tested variables, pairwise multiple comparisons were made among the individual levels of factors using the Tukey's HSD post hoc test at a significance level of $\alpha = 0.05$. The results of the analysis demonstrated that there was a significant difference ($p < 0.05$) within the repeated observations (number of active mites) between observations at 0 min and 30 min for all propolis types. Within the next 30 min observation time, a significant number of mites recovering from narcosis was observed at concentrations of 2% and 4% propolis for almost all propolis types (Fig. 5.1 a and b).

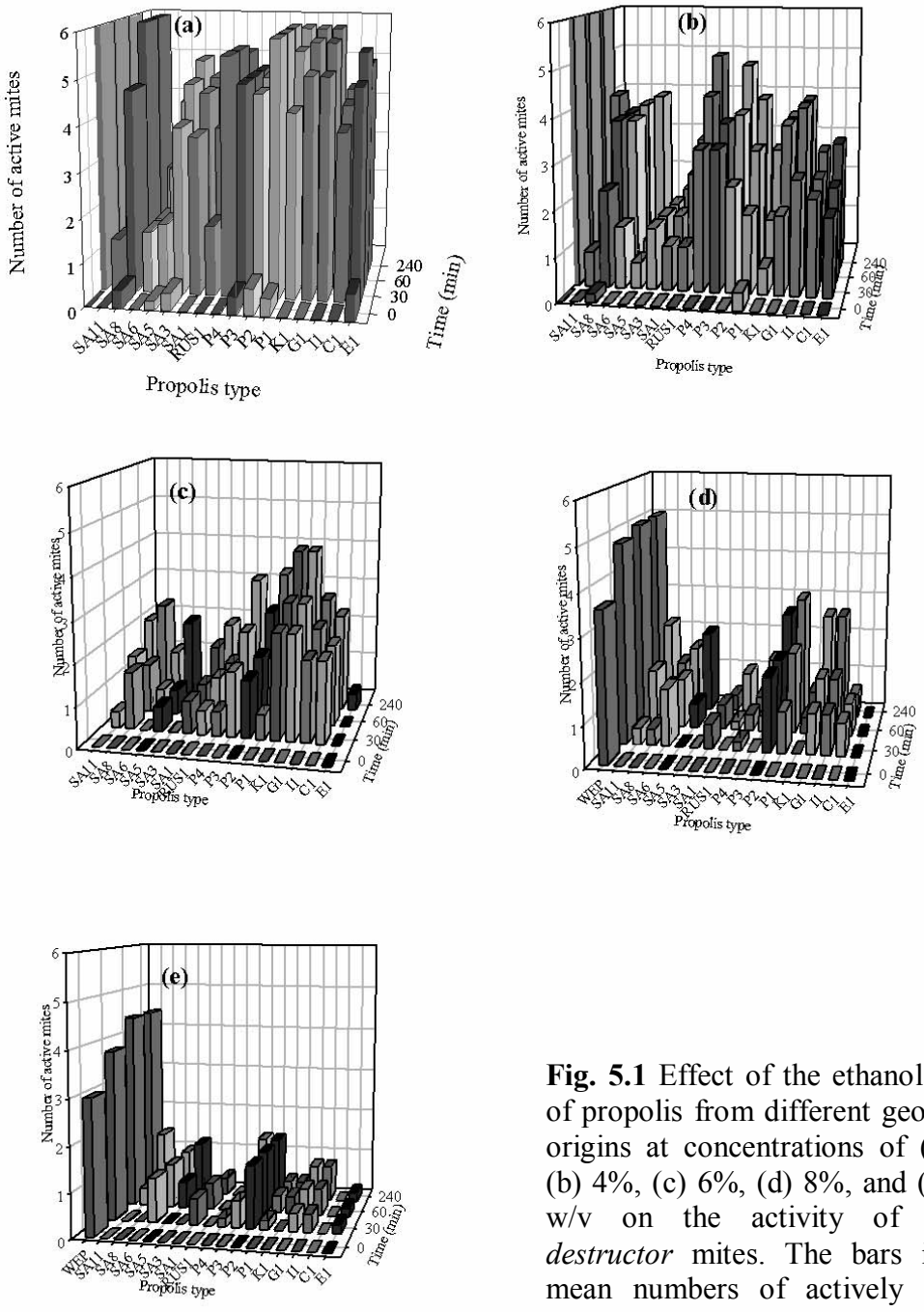

Fig. 5.1 Effect of the ethanol extract of propolis from different geographic origins at concentrations of (a) 2%, (b) 4%, (c) 6%, (d) 8%, and (e) 10% w/v on the activity of *Varroa destructor* mites. The bars indicate mean numbers of actively moving (non-narcotised) mites after treatment of 6 mites per experiment, n = 5. The 2%, 4%, and 6% WEP did not show any narcotic effect, thus, only those of 8% and 10% are displayed (Fig. 5.1 d and e).

At concentrations of 6% and 8% propolis, no significant changes in the activities of mites were observed between the 30th and 60th min of observation time for most propolis types. Significant activity changes for these 2 treatments were observed between the 60th and 90th or 90th and 120th min observation time (Fig. 5.1 c and d). In case of the treatment with 10% propolis, a significant activity change was observed only in the first 30 min; thereafter, practically no mite recovered from narcosis, with the exception of the samples SA5, SA11, P3, K1, G1, and I1, where a statistically significant ($p < 0.05$) activity change was observed between the observation times 30 to 120 min.

In case of the 8% WEP, activity change of the treated mites was observed between the 0 min and 30 min observation time; there was no significant change thereafter. A 10% WEP had a stronger narcotic effect compared to the 8%; hence mites were recovering from narcosis in the observation time interval of 30 to 60 min. There was no activity change observed for both concentrations after 60 min.

In addition to the significant difference at the level of individual between or within subject factors, significant differences also existed at the level of the product of the different subject factors (propolis type x concentration, propolis type x observation time, concentration x observation time, or propolis type x concentration x observation time). In order to discern clearly which group differs from the others, and at which concentration and observation time, a multiple pairwise comparison was done using the Tukey's HSD post hoc test at a level of significance of $\alpha = 0.05$. Following the Tukey's HSD post hoc test, the propolis samples were grouped into homogenous subgroups according to the statistically significant resemblance of the harmonic means of the narcotized Varroa mites (Table 5.2). The harmonic mean is occasionally used when dealing with averaging rates which are difficult to perform with the arithmetic mean, such as in the present case, as described by Croxton et al. (1967).

As can be discerned from Table 5.2, the water-extracted propolis (WEP) segregated from the rest of the group significantly ($p < 0.05$), and lies at the weakest end of the antivarroa spectrum, with a very low mean number of narcotized mites. Even though there was no clear segregation of the ethanol-extracted propolis (EEP) samples into independent groups based on their geographical origins, or bee subspecies that collected them, there was a tendency of the samples from Poland to segregate from the rest, with only the Italian sample (I1), the German sample (G1) and one of the South African samples (SA8) belonging to this group. The 4 South African samples (SA1, SA3, SA5 and SA6) belonged to the same homogenous subgroup together with the samples from Russia (RUS1) and Ethiopia (E1). The weakest homogeneity was

observed among the propolis samples from Colombia (C1), 2 from South Africa (SA8 and SA11), 1 from Poland (P4), and 1 from Kazakhstan (K1).

Table 5.2 Grouping of propolis samples into homogenous subgroups based on overall antivarroa activity

Propolis samples were categorised based on the overall homogeneity of the harmonic means of the number of inactivated *Varroa destructor* mites after treatment with various concentrations followed by repeated observation of mite activity. Repeated measures ANOVA, and Tukey's HSD post hoc test ($\alpha = 0.05$) were employed. The numbers in the cells indicate the harmonic means of the inactivated *Varroa* mites by each propolis sample with a 5 (concentrations) x 5 (number of replica) = 25 sample size.

	Homogenous subgroups based on α = 0.05							
Propolis type	1	2	3	4	5	6	7	8
WEP	0.62							
G1		3.27						
I1		3.36	3.36					
P2		3.37	3.37					
P1		3.39	3.39					
P3		3.68	3.68	3.68				
SA8		3.73	3.73	3.73	3.73			
P4			3.87	3.87	3.87	3.87		
SA11				4.16	4.16	4.16	4.16	
C1				4.20	4.20	4.20	4.20	
K1					4.31	4.31	4.31	
SA5						4.37	4.37	4.37
SA6						4.39	4.39	4.39
Rus1							4.48	4.48
SA1							4.53	4.53
E1							4.60	4.60
SA3								4.91
Significance	**1.000**	**0.317**	**0.165**	**0.135**	**0.054**	**0.135**	**0.445**	**0.109**

The homogeneity test run for the propolis types, in order to compare and put them into homogenous subgroups, was also run for the different concentrations of propolis to observe if any of the subsequent concentrations have similar antivarroa effects, and form a homogenous subgroup. This test with 17 (propolis types) x 5 (number of replica) = 85 (sample size) for each concentration displayed that no 2 or more concentrations formed a group, and that the antivarroa activity of each concentration was significantly different from that of the others ($p < 0.05$), i.e. no saturation effect was observed.

Further incubation of mites for longer than 4 h displayed no change in the mite's activity, thus, those that were narcotized up to this time were already dead. The number of dead mites at the end of each experiment was counted and presented in Fig. 5.2. Some propolis samples (e.g. G1, C1, P4, and K1) showed similar lethal effects at different concentrations, regardless of

differences in geographic origin. Generally, lethality increased with increasing concentration and 10 % EEP killed 85% to 100% of the treated mites.

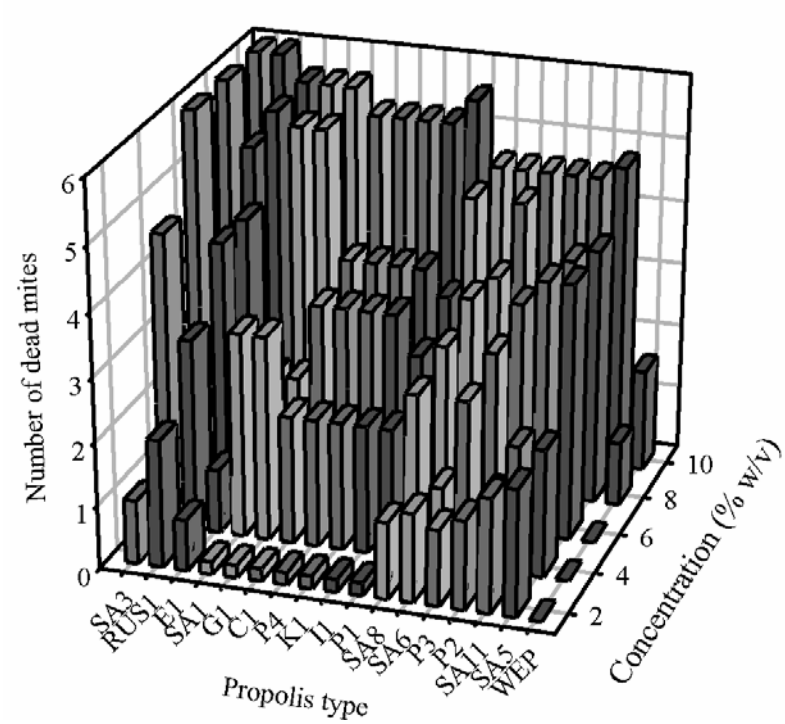

Fig. 5.2 Mortality of *Varroa destructor* mites after treatment with different concentrations of various propolis samples. The bars indicate mean numbers of dead mites at the end of the experiment. Each experiment involved the treatment of 6 mites, n = 5

The treatment of V*arroa destructor* with 4% EEP reduced the heat production rate significantly (paired sample t-test, $p < 0.05$) by 57.7 to 85.2% for all treatments, except the sample G1 which showed a reduction of 32.8%, still significantly different ($p = 0.01$) (Fig. 5.3 a). The effect of treatment with G1, displayed by the harmonic mean of percentage reduction in the heat production rate, was significantly lower than the other EEP treatments, and it formed a homogenous subgroup only with the 10% WEP (Table 5.3 b). The 10% WEP reduced the mites' heat production rate significantly ($p = 0.02$) by 23.2%. The control treatment (55% ethanol) had no significant effect on the heat production rate ($p = 0.18$) with a reduction by 7.8 ± 2.1%. A one-way ANOVA followed by the Tukey's HSD post hoc test, and categorization of the treatments into homogenous subgroups, based on the significant homogeneity of the harmonic mean reduction of heat production rate due to treatment, is shown in Table 5.3 b. As can be discerned from this table the control segregates from the rest, with a very low and insignificant percentage reduction of heat production rate. WEP and G1 form a subgroup (with a significant resemblance level of $p = 0.25$) with the lowest harmonic mean among the propolis treatments,

indicating the weakness of their antivarroa activity. The rest of propolis samples belong to the same subgroup with a significance level of $p = 0.646$ and a higher antivarroa activity.

It has to be born in mind that the heat production rate after treatment with propolis is the result of metabolism of the survivor mites. Though it is meaningful to consider the total drop in the heat production rate due to treatment with propolis, since this reflects the extent to which the treatment group as a whole suffered from the treatment, it is also desirable to discern the extent of weakening of the survivor mites. Comparison of the specific heat production rate before treatment with that of only the survivor mites after treatment was conducted by separating the survivors at the end of the calorimetric experiment, and dividing the heat production rate after treatment by the weight of the survivors. Immediately after treatment mites were normally narcotized for a length of time that depended on the type and concentration of propolis, and they produced a negligible amount of heat during this period. During the course of incubation time the mites were recovering from narcosis and started moving, subsequently increasing their heat production rate. The course of heat production rate increased with time until all survivor mites recovered from narcosis, and start metabolizing with the potential they could achieve after treatment. After this point the *p-t* curve did not ascend anymore and remained at a certain level forming a plateau phase. The heat production rate at the plateau phase was recorded for ca. 2 to 4 h, depending on the stability of level of the *p-t* curve, after which the experiment was stopped to avoid starvation and death of mites. The survivor mites were separated from the dead ones and weighed. If the experiment has been run longer, the curve would have started declining due to mite starvation and death. Only the heat production rate at the stable plateau phase was considered in the calculation of specific heat production rate of the survivor mites.

As there were no dead mites and hence the number of survivor mites equals the total number of mites after treatment with the control (55% ethanol), the specific heat production rate after treatment was the same whether considered per weight of total number of mites or per weight of the survivor mites. Though there was a drop of the specific heat production rate by 7.8 ± 2.0% due to the control treatment it was statistically insignificant (Paired sample t-test, $\alpha = 0.05$). Even though the treatments with G1, C1, E1, K1, SA1, SA3, SA5, and WEP caused a reduction of the specific heat production rate when considered per weight of the total number of mites, the specific heat production rate of the survivor mites remained unaffected (Fig. 5.3 b and Fig. 5.4). This indicates that the drop in the specific heat production rate was caused by the death of some mites, but not due to the narcotizing and weakening of the survivor mites.

Table 5.3 Grouping of propolis samples into homogenous subgroups based on the mortality and reduction of heat production rate of *Varroa destructor*

Subgroups of propolis based on treatments with a 4% EEP and a 10% WEP, and observation of (a) the mean percentage of dead mites at the end of the calorimetric experiment, and (b) the percentage reduction of heat production rate. (n = 3, 25 to 30 mites per experiment). The harmonic mean values are displayed. One-way ANOVA and Tukey's HSD test (α = 0.05) were employed.

(a)

	Subgroups based on α = 0.05			
Propolis type	1	2	3	4
control	0.00			
P2		22.67		
P4		24.11		
WEP		26.2		
G1		33.46		
P1		33.63		
SA5		37.33		
I1		39.13	39.13	
SA8		42.67	42.67	42.67
SA11		43.53	43.53	43.53
SA6		45.83	45.83	45.83
P3		45.55	45.55	45.55
RUS1		49.38	49.38	49.38
K1		54.25	54.25	54.25
C1		56.32	56.32	56.32
E1		58.45	58.45	58.45
SA1				76.29
SA3				78.85
Significance level	**1.000**	**0.074**	**0.099**	**0.083**

(b)

	Subgroups based on α = 0.05		
Propolis type	1	2	3
control	7.80		
WEP		23.06	
G1		33.57	
C1			54.67
SA5			56.09
I1			59.33
K1			60.59
SA11			60.65
E1			66.53
P1			69.48
SA8			70.00
P3			71.67
P4			76.59
Rus1			77.45
SA6			77.49
SA3			78.08
SA1			81.59
P2			85.24
Significance level	**1.000**	**0.250**	**0.646**

The treatments with P2 and P4 caused significantly higher reduction of the specific heat production rate of the survivor mites compared to the other treatments (one-way ANOVA and a Tukey's HSD test, $p < 0.05$), and they formed a homogenous subgroup ($p = 0.36$). Comparison of the percentage reduction of the survivors' specific heat production rate with that of the percentage mortality data (cf. Fig. 5.4 with Fig. 5.5) displays that the treatments with P2 and P4 caused less mortality. Therefore, their effect is mainly narcotizing and weakening the mites rather than killing them, at least at this concentration.

Comparison of the differences in the percentage reductions of the specific heat production rates achieved by the various treatments showed that there is a statistically significant difference (one-way ANOVA, Tukey's HSD post hoc test, $p < 0.05$) between 4% EEP and 10% WEP of G1 on one hand, and that of the other EEP treatments on the other hand (Table 5.3 b). The difference between the reductions of specific heat production rate by 4% EEP and 10% WEP of G1 was not significant ($p = 0.25$), showing the weakness of the water soluble components of propolis; a 10% WEP possessing an activity comparable to a 4% EEP. The percentage reduction of specific heat production rate by the control treatment was significantly lower than the rest of the treatments (one-way ANOVA, Tukey's HSD post hoc test, $p = 0.01$).

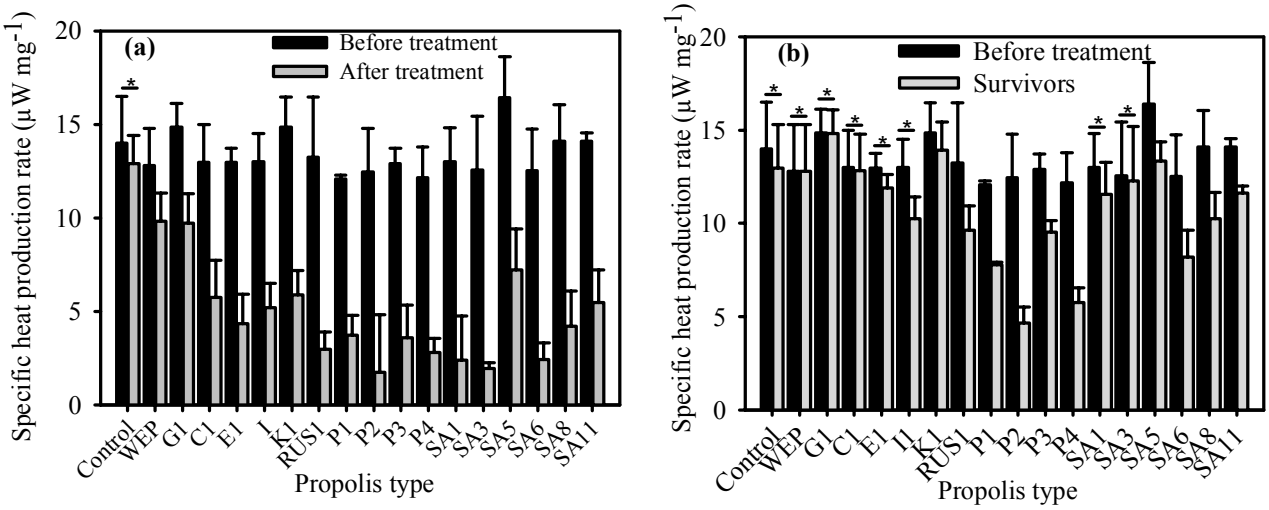

Fig. 5.3 Effect of treatment of *Varroa destructor* mites with a 4% EEP and a 10% WEP on the specific heat production rate of (a) total mites after treatment (b) only the survivor mites after treatment. Mean ± S.D., n = 3, 25 to 30 mites per experiment, * = non-significant difference before and after treatment (paired sample t-test, $\alpha = 0.05$).

Comparison of the percentage mortality of mites and the percentage reduction of specific heat production rate due to treatment with the different propolis samples displayed that the

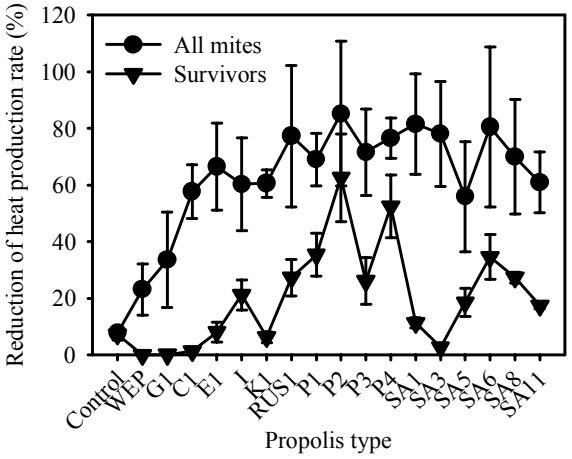

Fig. 5.4 Percentage reduction of mass specific heat production rate calculated based on (a) the weight of all treated mites and (b) the weight of only the survivor mites after treatment with 4% EEP and 10% WEP. n = 3, 25 to 30 mites per experiment, control 55% ethanol.

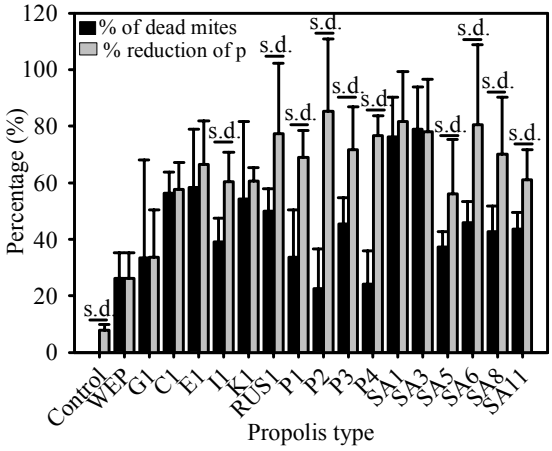

Fig. 5.5 Comparison of the percentage reduction of heat production rate (p) and percentage of dead *Varroa destructor* mites at the end of the calorimetric experiments after treatment with 4% EEP of different propolis samples and with 10% WEP of G1. n = 3, 25 to 30 mites per experiment. The control involved treatment with 55% ethanol. s.d. = significant difference between percentage reduction of heat production rate and percentage of dead mites (t-test, α= 0.05). ANOVA test results among the different treatments are displayed in Table 5.1 a and b.

percentage of dead mites was either equal to or less than the percentage reduction of the specific heat production rate (Fig. 5.5). In treatments where the percentage of dead mites was not significantly different (t-test, $p > 0.05$) from the percentage reduction of the specific heat production rate (WEP, G1, C1, E1, K1, SA1, SA3), there was no significant difference between the specific heat production rates before treatment and those of survivor mites (cf. Fig. 5.4 with Fig. 5.5). In such cases, the specific heat production rate of the survivors remained unaffected as before treatment, or it was reduced insignificantly, since the drop in the specific heat production rate was caused by the death of some mites. A one-way ANOVA test on the percentage of dead mites after treatment with different propolis samples followed by Tukey's HSD post hoc test, and grouping the treatments into homogenous subgroups based on the significant resemblance of their harmonic means is displayed in Table 5.3 a. As can be seen, the control does not belong to any other subgroup, due to lack of lethal effect, and the treatments with SA1 and SA3 segregate on the other end of the spectrum with maximum lethality.

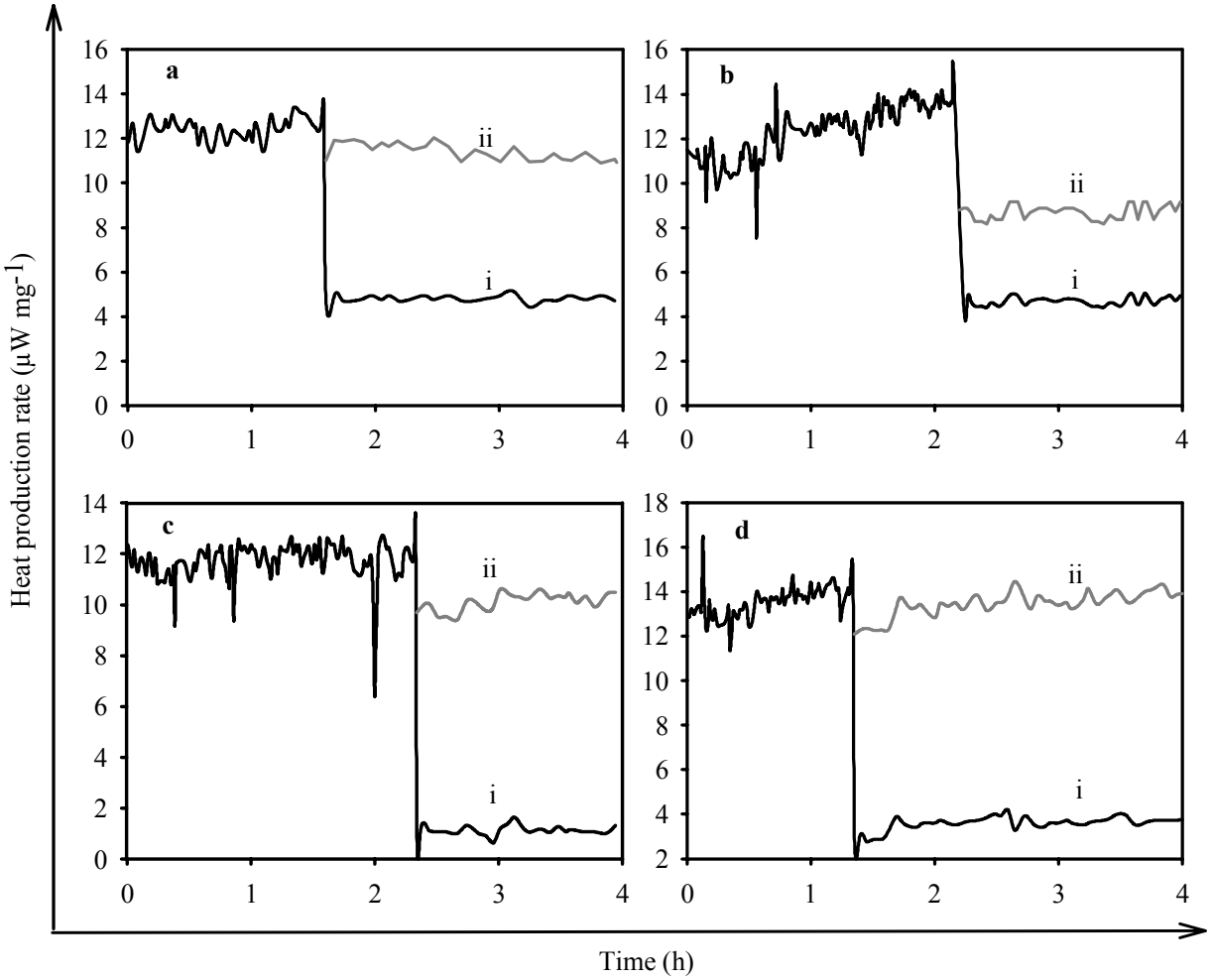

Fig. 5.6 Effect of treatment of *Varroa destructor* mites with 4% EEP of (a) K1, (b) P1, (c) SA1, and (d) SA11 on the structure of the *p-t* curve and on the mass specific heat production rate calculated based on the (i) weight of the total number of mites treated and (ii) weight of only the mites that survived the treatment. 30 mites were used per experiment.

The treatment of mites with 4% propolis not only dropped the specific heat production rate (calculated based on both the weight of total number or only survivor mites), but also changed the structure of the *p-t* curve (Fig. 5.6), smoothing it considerably.

The electron microscopic pictures (Fig. 5.7) show that the treatment of mites with propolis destroyed their cuticle on the different parts of the body, both dorsally and ventrally. The ventral side seems to be highly vulnerable due to the thin layer of cuticle, compared to the thick and more resistant layer on the dorsal side.

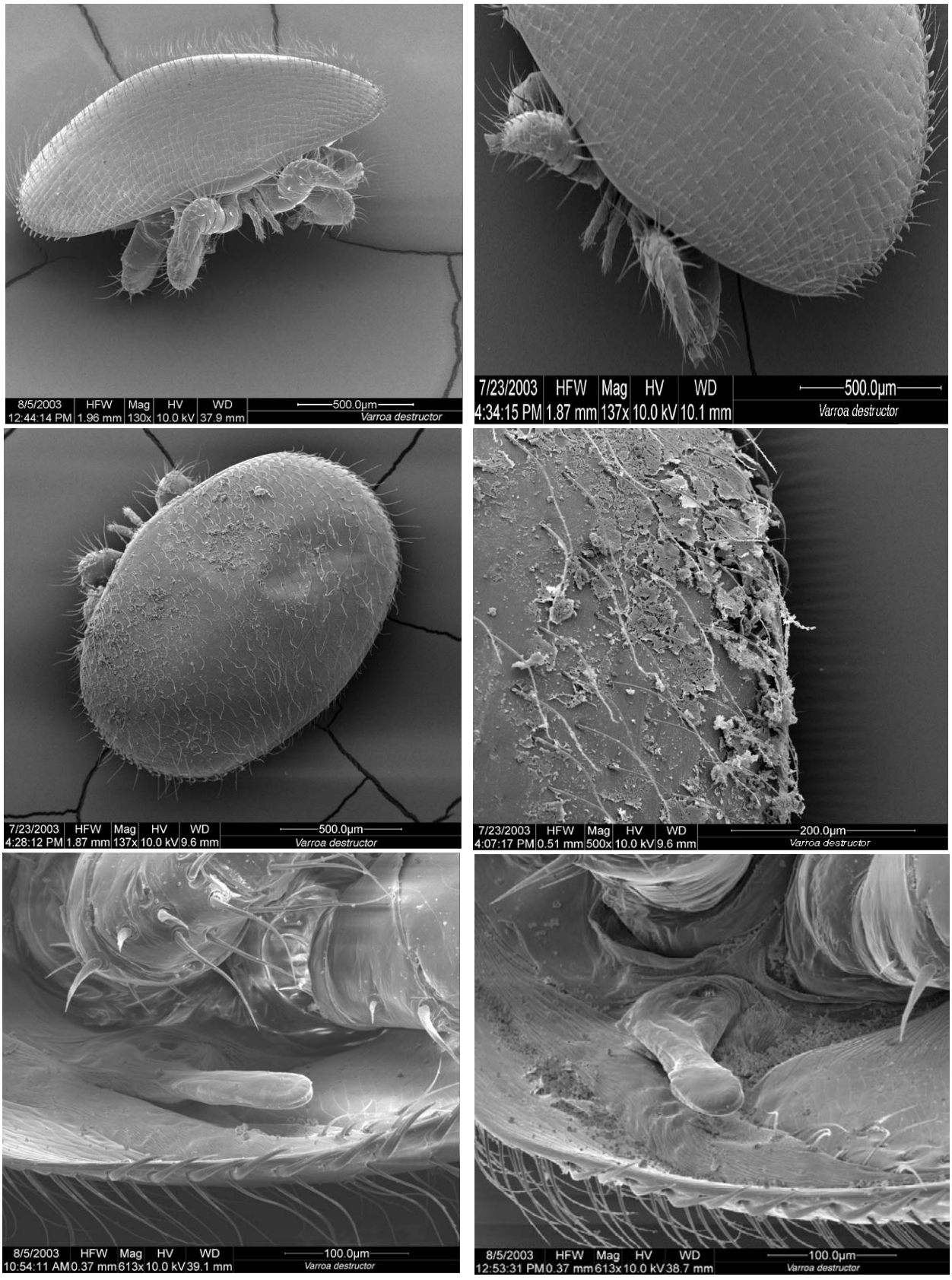

Fig. 5.7 Scanning electron microscopic pictures of *Varroa destructor* mites after treatment with 10% EEP of G1: (a) a control mite, (b) detailed view of the control mite, (c) a treated mite, (d) detailed view of the treated mite, (e) ventral side detailed view of an untreated mite, and (f) ventral side detailed view of a treated mite. (FEI Quanta 200)

5.5 Discussion

The antivarroa action of water-extracted propolis (WEP) was weaker than that of the ethanol-extracted (EEP) ones. The weak biocidal action of WEP compared to EEP was already demonstrated against different types of microbes (Neunaber 1995). The lack of antivarroa action of weak concentrations of WEP (2%, 4%, and 6% w/v) could explain why propolis is not active against mites in the beehive environment. As the WEP makes up a small proportion of the total propolis extract, 3.5% w/w (own result), 2.6 to 6.3% w/w (Spiridonov et al. 1992, Neunaber 1995), 2.0 to 4.0% for Chinese propolis and 6.0 to 14.0% for Brazilian propolis (Miyataka et al. 1997), it is not surprising to find Varroa mites in the beehive, where propolis forms a thin layer on all internal walls and comb cells. The antivarroa actions of higher concentrations of WEP indicate that these components of propolis, in addition to other propolis components such as the volatile ones, could contribute to the Varroa resistance by some bee species. Although the possible insecticidal and varroacidal actions of propolis in the beehive have already been postulated (König and Dustmann 1988, Amrin et al. 1996) until now no experimental evidence existed. If propolis is to play a role in the beehive environment, the 2 groups of substances that are responsible for this action could be the water soluble and the volatile/essential oil components. The Varroa narcotizing and varroacidal actions of WEP $\geq$ 8% could be proof that in geographical locations where bees collect propolis with higher proportion of water soluble components, propolis could play a role in keeping the Varroa population lower. The majority of components of propolis which are soluble only in non-polar solvents do not have roles in the beehive environment due to insolubility in water.

The strength of narcosis imparted on the mites and subsequently the time needed to recover was dependent on the concentration of propolis. A higher concentration of propolis resulted in a longer time period required for the mites to recover from narcosis, if they were to recover at all. At concentrations above a certain threshold value the antivarroa action of propolis becomes lethal, rather than recoverable narcosis. Strong concentrations, such as 10% E1, Rus1, and SA3 resulted in the immediate death of all treated mites and no mite was observed recovering from narcosis. In addition to variation in the strength of antivarroa effects among concentrations, differences were also observed among samples of different origins at a certain concentration. The difference in the strength of narcosis among different samples could be an indication of quantitative and/or qualitative variations in the narcotic and lethal antivarroa agents they possess. Propolis samples that differ in overall chemical composition may show similar antivarroa effects as far as the substances they possess, which, even if different, have comparable effects.

Based on literature data of propolis from different geographic origins (Marcucci 1995, Kujumgiev et al. 1999, Bankova et al. 2002) it is reasonable to state that the samples differ in their chemical composition. The extent of similarities in the chemical composition of propolis samples depends on the type of plants visited by the bees, which is in turn determined by the geographical location (Ghisalberti 1979, Marcucci 1995, Markham et al. 1996, Miyataka et al. 1997, Burdock 1998, Kujumgiev et al. 1999, Bankova et al. 2000, Bankova et al. 2002). Thus, bees in tropical regions may have a completely different propolis source than bees in the temperate regions. Tropical propolis such as E1 and C1 could, therefore, have completely different chemical composition than temperate propolis. Though the specific chemical make-up of the plant resins collected by the bees differs based on the plant species visited, the general framework of bioactivity could be more or less similar (Kujumgiev et al. 1999), since the purpose for which plants secret resins resemble at least partially. Resin is mainly secreted by plants in order to seal wound, to stop sap loss, to protect wounds from infection by microbes, to protect pollen against infection (it is coated with resin), to stop germination of seeds, and to inhibit sprouting of bud while frost (Ogren 1990). As in case of the use of resin by plants, the use of propolis in the beehive is more or less the same, regardless of the geographic location of the beehives, and this includes defending the hive interior from invading bacteria and fungi and even invading larvae (Ghisalberti 1979, Lisowski 1984, Marcucci 1995).

Even though various samples showed different patterns of activity when considering the length of narcosis, and the number of mites that recover from narcosis in a given period of time, the final effect (lethality) achieved by a certain concentration of most samples was comparable (cf. Fig. 5.1 a to e and Fig. 5.2). This could be an indication of identical functions regardless of origin.

Although the sample G1 did not show significantly lower narcotic or lethal effects at various concentration levels with the Petridish bioassay experiments, its effect on the reduction of specific metabolic rate was inferior to all other EEPs at 4% w/v. This could be due to the fact that the metabolic rate of survivors remains unaffected, apart from the reduction caused by death of certain mites (Fig. 5.3 b).

Comparison of the lethality and reduction of the metabolic rate achieved by a sample shows that the higher the lethality, the higher the reduction in the specific heat production rate, calculated based on weight of the total number of treated mites. In a few cases such as SA1, SA3, K1, C1, G1, E1, and WEP the percentage of dead mites was not significantly different from the percentage reduction of heat production rate (Fig. 5.5). In these cases the reduction of specific heat production rate was mainly due to the death of some mites, but not due to the

weakening of the survivors, since reduction of the specific heat production rate of the latter was insignificant and lay near to the zero line (Fig. 5.4). In a number of cases, however, low mortality rates were accompanied by high reductions of the specific heat production rates. This could be due to the fact that the survivor mites were highly weakened and, thus, their metabolic rate was reduced considerably (cf. Fig. 5.4 with Fig. 5.5).

In addition to showing the general antivarroa effect of propolis, which includes death of certain mites and weakening of the rest, the calorimetric method also displays the extent to which the survivors are weakened, and, hence, their metabolic rate is reduced. The treatment with propolis affected the structure of the *p-t* curves, which is the result of the movement and activity of the mites. After treatment with propolis, the *p-t* curve was smoothed (Fig. 5.6) mainly due to the narcotic effect and, thus, the inability of the mites to move actively as they could do before the treatment.

The destruction of the cuticle, creating structural deformities on the mite's protective surface, could facilitate the entry of the biocidal agents into the mite's body, leading to narcosis and consequential death. Since the ventral side is covered with a thin layer of cuticle it can easily be destroyed as compared to the dorsal hard and thick cuticular layer.