1.0 General Introduction

In this thesis three main subjects are integrated: the impact on honeybees and the possible means of control of the honeybee parasites and pests *Varroa destructor* (Anderson and Trueman), and *Galleria mellonella* L.; the natural bee product propolis, and its biocidal use; calorimetry as a technique applied in the investigations of metabolic rates and the sublethal effects of propolis. As the thesis is an interdisciplinary approach involving the mentioned fields, it is important to give concise introductions to the main subjects. The intention of these introductions is that a calorimetrist reading this thesis can gain some general ideas about the other subjects in the area of bee research. The same holds true for a bee researcher to whom, in most cases, the what about and the working principles of calorimeters are not familiar, reading this thesis without a proper introduction of calorimetry may make it difficult to grasp what is being conveyed.

1.1 Varroa mites as parasites of honeybees

The hive of honeybees with its constantly maintained optimal temperature, humidity, and carbon dioxide level, year round ample availability of the host bees, protinacious (pollen), carbohydrate (honey), and wax foods, is a suitable habitat for a diverse array of parasites and pathogens (Bailey and Ball 1991). Some of the most common parasites and pathogens of the honeybees include viruses (acute paralysis virus – APV, deformed wing virus – DWV, and sack brood virus - SBV), bacteria (*Paenibacillus larvae larvae* - American foulbrood, and *Melissococcus pluton* – European foulbrood), fungi (*Ascosphaera apis* – chalkbrood, and *Aspergillus flavus* – stonebrood), protists (*Nosema apis* - nosema disease, and *Malphigamoeba mellifica* - amoeba disease), mites (*Varroa destructor*- varroosis, and *Acarapis woodi* – tracheal mites), and insects (*Galleria mellonella* – the greater wax moth, and *Aethina tumida* - the small hive beetle). Among the different parasites and pathogens mentioned, the parasitic mite *Varroa destructor* (Anderson and Trueman, formerly called *Varroa jacobsoni* Oud.) is becoming a global concern affecting the beekeeping industry based on *Apis mellifera* L. (Boecking and Spivak 1999), and it is attracting the attention of researchers to circumvent the perish of the honeybee. It is not only the beekeeping industry that suffers from loss of the honeybees; rather the crop agricultural sector is also being hit by this problem, because most plants are dependent on bees for pollination. It is estimated that 80% of all crop insect pollinations are accomplished by honeybees (Benedek 1985).

The infestation of *Apis mellifera* L. by *Varroa destructor* reportedly originated nearly half a century ago (Smirnov 1978, Crane 1979, Matheson 1995), when the mites transferred to *A.*

mellifera colonies that had been introduced into the home range of *A. cerana* Fab., the mite's original host. *Varroa destructor* is an obligate ectoparasite that feeds on the hemolymph of bees both in the capped developmental stage and on adults, but reproduces only in the capped worker and drone brood of *A. mellifera,* and only in the drone brood of *Apis cerana*. In its original host, the Asian honeybee *Apis cerana*, a host-parasite relationship has evolved that rarely damages the host (Anderson and Trueman 2000). In the case of *A. mellifera* colonies, however, mortality from *V. destructor* can reach up to 100% within two to five years, if mite control methods are not implemented (De Jong 1997). Additionally, high mite populations were observed to be associated with increased incidences of viral infections (Ball 1994), lower weight at hatching, and shortened life span of the adult bees (De Jong et al. 1982), as well as deformed wing and shortened abdomen.

The vertical transmission of Varroa mites from individuals of the parent to those of the offspring colony involves the formation of a daughter colony with parasites from the parent colony after swarming, or the splitting of parent colony by the beekeeper.

The extent of the problem of varroosis is alarming mainly due to the very high spread potential and debilitating action of the parasitic mites. The very close contact between bees in a colony facilitates the easy intracolony spread of the parasite among individuals within a generation (horizontal transmission). This adds up to the likely demise of the colony, should even a single member of it is infested. The very high horizontal intercolony dispersion potential of *V. destructor* can be attributed to at least two main factors: firstly, because of activities of beekeepers moving colonies from place to place for commercial and pollination purposes; secondly, due to intercolony drifting and robbery of infested bees. The intercolony drifting of infested bees and the spread of varroosis is worsened by the repercussion effect of the weakening of colony-state factors, and, thus, behavioural change of the individual workers by the parasitic mite (Downey et al. 2000). Several stress events, such as wax deprivation (due to its insufficient production as a result of underdeveloped glands of the infested bees), depletion of nectar and pollen stores, and worker loss (Winston and Fergusson 1985) induce resource gathering responses in honeybee colonies. Such responses include an increased number of foragers, accelerated task ontogeny (i.e., earlier onset of foraging flights), or a greater effort by individual foragers (visiting more flowers or carrying larger pollen, nectar, or propolis loads) (Schmid-Hempel et al. 1993). Among the different responses of the colony to the stress imposed on it, the precocious foraging (accelerated task ontogeny) contributes to the increased horizontal transmission of parasites from an infested colony to another one by the increased drifting of parasitized and weakened workers. It was confirmed by Schneider and Drescher (1988) that

worker bees parasitized by Varroa mites during their pupal development start flying earlier, and the rate of drifting of such bees was found to be very high compared to drifting by non-infested bees. Bowen-Walker and Gunn (2001) explained the possible reason for the higher drifting rate in infested colonies to be due to the fact that by flying earlier in their lives, infested bees start out nest activities before their memory/orientation is fully developed, leading to their disorientation and loss.

1.1.1 Biology of *Varroa destructor* mites

The female *V. destructor* mite is brown to reddish-brown in colour, measuring 1.1 to 1.2 mm in length and 1.5 to 1.6 mm in width (about the size of a pinhead) (Fig. 1.1 a and b). Males are smaller, about 0.7 mm by 0.7 mm, and light tan in colour. Even though the female mite parasitizes larval, pupal, and adult developmental stages of the honeybee, reproduction takes place only in the capped brood developmental stage (Infantidis 1983, Martin 1994, Steiner et al. 1994). This reproduction lasts 12 days in worker and 15 days in drone brood (Moritz 1985, Le Conte and Cornuet 1989). Outside the capped brood, the female Varroa mites live on adults, mostly on nurse bees, using them mainly as short term hosts and for dispersal (phoresy); for this reason mites on adult bees are called phoretic mites. It was forwarded by Hoppe and Ritter (1989) that Varroa mites prefer young “house” bees to older worker bees, probably due to the lower titer of the Nasonov gland pheromone geraniol, which strongly repels the mite. When on adult bees the mite fits itself beneath the bee’s abdominal sclerites, lessening transpirational water loss, and reducing the vulnerability to grooming and dislodgement during host activity (Sammataro et al. 2000). The dorsoventrally compressed body shape of the mite allows it to fit properly into the intersegmental groove, at the same time accessing the soft integument that can be pierced by the mite’s chelicerae, enabling it to feed on the bee’s hemolymph. Males are not able to pierce even the soft integument of the brood stage, since their mouth part is modified for sperm transfer (Frazier 2000). As a result, male mites are dependent on the hole made by the female mites to suck hemolymph from the brood stage. In addition to that, the body structure of male mites is not optimally compressed to fit under the abdominal sclerites of the adults. For these reasons the lifespan of male mites is restricted only to the capped brood developmental stage.

One or sometimes more foundress Varroa mites enter the prepupal stage of a worker brood 20 h, and a drone brood 45 h before cell capping in order to reproduce (Boot et al. 1991), and start feeding on the brood and its reserve food. The time at which the mother mites enter the uncapped brood indicates the period of attractiveness of the brood to mites, since only brood of a

particular age is attractive to them (Fries et al. 1994). Drone brood was found out to be more attractive to Varroa mites than worker brood (Fuchs 1990). The higher preference of drone brood to worker brood by Varroa mites was considered to be the result of a combination of several factors. These factors include chemical attractants, such as fatty acid esters secreted by the larvae, and present on the cuticle of drone brood at higher quantity and/or quality than in worker brood, at the mite attractive age of the brood (Le Conte et al. 1989, Trouiller et al. 1991, Beetsma et al. 1999, Sammataro et al. 2000). Non-chemical factors include longer period of pre-capped Varroa attractive stage (Fuchs and Müller 1988, Infantidis 1988, Boot et al. 1991) as well as bigger size of the drone brood cell, which increases the chance of encounter (Sammataro et al. 2000). In addition to that, broods that are big and grown up to the rim of the brood cell are more attractive than broods far from the rim (Beetsma et al. 1999).

After entering uncapped brood cells the mites hide from the removal action of the nurse bees by submerging into the liquid brood food until cell capping. While in the submerged state, they use their peritremes (Fig. 1.1 c, and d) which protrude snorkel-like above the liquid food for respiration (Donzé and Guerin 1997). Mites emerge out of their concealment after brood capping. A foundress mite lays its first egg, which develops to a haploid male (n = 7), 60 h after cell capping, and the subsequent eggs are laid in intervals of 30 h and develop to diploid females (2n = 14) (Steiner et al. 1982, De Ruijter and Pappas 1983, Infantidis 1983, Rehm and Ritter 1989).

The male mite requires 6.9 days to reach the adult stage whereas a female needs only 6.2 days (Rehm and Ritter 1989). The male mite is already a mature adult by the time its sisters reach maturity, and it copulates with all of its adult sisters as often as possible, to ensure fertilization, before the bee emerges as a callow bee (Sammataro 2000). After the bee has hatched and left the cell with the mature female mites, the male and nymph stages of the female mites die of starvation. The number of female mites that reach maturity is directly affected, among other factors, by the length of post capping developmental period. Since the drone brood has a longer post capping developmental period of 15 days, compared to the 12 days for workers (Moritz 1985, Le Conte and Cornuet 1989) higher numbers of female mites emerge as adults from drone than from worker cells.

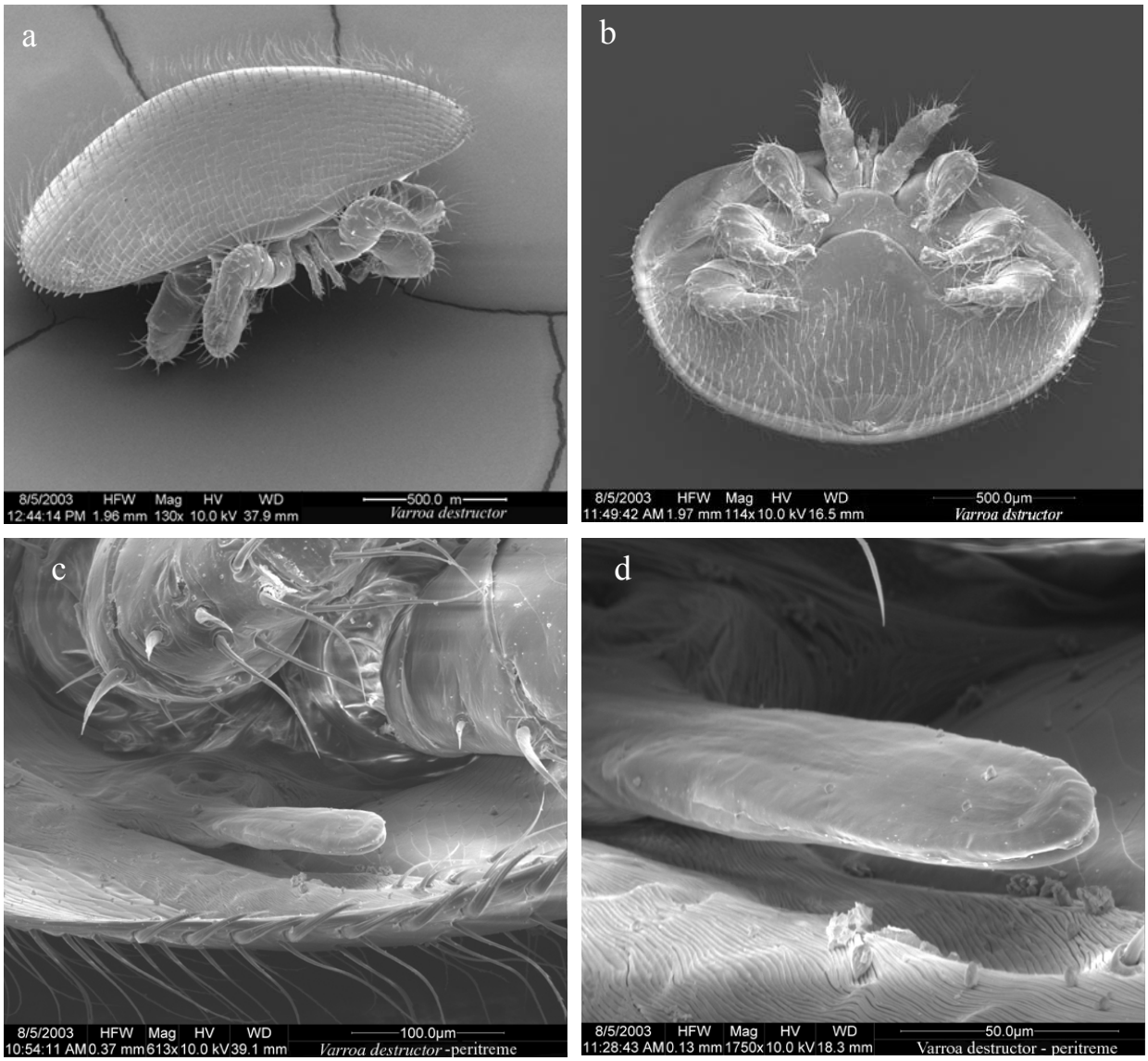

Fig. 1.1 Scanning electron microscopic pictures of the female *Varroa destructor,* (a) dorso-frontal view, (b) ventral view, (c) the breathing structures stigma and pertitreme, and (d) an enlarged peritreme with the slit. (FEI, Quanta 200).

1.1.2 Defence mechanisms of *A. mellifera*

In general, the defence mechanisms of honeybees to protect themselves from pathogens and parasites include (a) a constitutional defence - the chitinous exoskeleton, (b) cellular and humoral defences - haemocytes, and enzymes and antimicrobial factors, respectively, (c) a physiological defence –the proventricular valve that filters ingested spores, and (d) a behavioural defence - activity of the honeybees to keep themselves, their nest mates, and their hive clean; an important mechanism to stop the spread of pathogens such as American foulbrood, chalk brood, and parasites. The behavioural defence mechanism is of special interest from the perspective of the bee's defence mechanism against parasites such as *Varroa destructor.*

1.1.3 Behavioural defence of *Apis mellifera* against *Varroa destructor*

The behavioural defence mechanisms of *Apis mellifera* that enable it to reduce the population of *V. destructor* mites are hygienic and grooming behaviours (Boecking and Spivak 1999). The expression of these two behavioural traits in *A. mellifera* is far lower compared to that in *Apis cerana,* the original host of Varroa mites (Peng et al. 1987, Boecking and Ritter 1993, Shimanuki et al. 1994). The higher grooming and hygienic activity of *A. cerana* combined with the non-fertility of mites in worker brood endowed tolerance to this bee species enabling it to live in equilibrium with Varroa mites (Fries et al. 1994, Boecking and Spivak 1999).

Regardless of the effort of a colony to keep its hive clean and free of parasites and pests, which actually varies from race to race (Buchler 1994) and is influenced by climatic conditions (De Jong et al. 1984, Kraus and Velthuis 1997), beekeeping with the western honeybee *A. mellifera* is being highly jeopardized by *V. destructor*. Though some fragmentary and anecdotal reports exist about the tolerance of some *A. mellifera* colonies to *V. destructor* from different regions, the only race of the western honeybee which is confirmed to be tolerant to varroosis and does not need human interference is the Africanized honeybee (De Jong et al. 1984, De Jong 1996, Medina and Martin 1999). Factors that are involved in the resistance of varroosis by Africanized honeybees include reduced mite fertility (Martin et al. 1997), higher offspring mortality (Medina and Martin 1999), smaller brood cell and hence limiting space (Message and Gonçalves 1995), shorter post capping developmental period (Moritz 1985), and food (pollen) availability (Moretto et al. 1997). In addition to these, behavioural factors of the bees such as hygienic (Corrêa-Marques and De Jong 1998) and grooming (Moretto et al. 1993) behaviours were deemed crucial.

The threat of *V. destructor* to the non-Africanized western honeybees is so alarming that colonies have to be somehow treated or manipulated in order to reduce the population size of Varroa mites and to save the colony from dying out.

1.1.4 Control methods of *Varroa destructor*

Different methods of treatment of a colony are available nowadays, even though some of them are ineffective and others have limitations due to their effects on the bees or the beekeeper. The methods of Varroa prevention and control include biotechnical, biological, and chemical methods.

1.1.4.1 Biological methods

The biological Varroa control methods involve the use of the bee's biology, perhaps its natural resistance against mites. The desirable features of bees that can be selected to establish a resistant colony include higher hygienic and grooming activities, shorter post capping periods, low attractiveness of brood to mites, and low mite fecundity factors. The selection and establishment of resistant colonies is the best and cheapest method of control of varroosis since the bees themselves deal with Varroa mites. Achievement of this control method is, however, taking longer time and short term solutions, such as biotechnical or chemical methods have to be used in the meantime to stop colony death.

1.1.4.2 Biotechnical methods

Biotechnical methods of mite control utilize the principle that mites inside a capped brood are trapped and hence can be removed from the colony. The drone brood, which is often unwanted by the beekeeper, can be used as a trap comb. In the period of absence or scarcity of drone brood, worker brood can also be used as a trap. It is, however, undesirable to destroy worker brood with the trapped mites; the mites have to be killed selectively. The selective killing of mites can be done at a high temperature (44 °C) (Rosenkranz 1987, Engels 1994), and with the use of formic acid (Fries 1991, Calis et al 1998)

1.1.4.3 Chemical methods

The chemical methods of mite control involve various methods of application and ways of dispersal of the acaricides, which are determined by the nature of the chemicals being used. The methods of application include: (a) hanging impregnated plastic strips between combs in the brood chamber. The chemicals are distributed among members of the colony by contact of some bees with the impregnated strip and subsequently with their nest mates. The crowded life style and close contact among bees is responsible for the distribution of acaricides applied this way. This method is used to apply Bayvarol™ (flumethrin as the active ingredient - a synthetic pyrethroid), Apistan™ (fluvalinate as the active ingredient - a synthetic pyrethroid), and Apivar™ (amitraz as the active ingredient). (b) Emulsion or solution in water trickled into bee spaces between combs. Perizin™ emulsion (with coumaphos, an organophosphate, as the active ingredient) and Apitol™ solution (with cymiazol hydrochloride as an active ingredient) are applied this way, and distributed among individuals in a colony by grooming and trophallaxis (food exchange between bees). Organic acids such as oxalic and lactic acids are also trickled on bees in sugar syrup. (c) Feeding acaricides to bees with sugar solutions so that it is distributed by

trophallaxis. This method is used in the application of Galecron™/K79 (with chlordimeform as the active ingredient). (d) Smouldering of impregnated cardboard strips in a sealed hive. This method is used to apply Folbex VA™ (with bromopropylate as the active ingredient). Spreading of the active ingredient in the beehive is achieved by combustion. (e) Placing impregnated cardboard on top of the brood combs. Essential oils such as thymol, neem oil, and others are applied this way under the trade name Api-Life VAR™. Evaporation is the means by which distribution is achieved in the beehive. The mechanism by which essential oils act was supposed to be lethality at higher concentrations, and interference with the olfactory senses and orientation of mites at sublethal concentrations (Kraus et al. 1994). Since higher concentrations may also affect honeybees, it may be desirable to use sublethal doses of essential oils. These lower concentrations interfere with the chemoreceptors of the mites, making them unable to locate brood cells to invade and reproduce (Kraus et al. 1994), thus, falling to the bottom and dying of hunger. (f) Evaporation of a solution. Nowadays formic acid is applied in a colony by placing it in an evaporator, which allows the gradual evaporation of the acid.

Though some of the chemicals used for the control of Varroa mites in different parts of the world are toxic to the honeybees and humans, the chemical method of treatment is the only effective and non laborious method presently available to the beekeeper. Several researchers are focusing on the potential use of natural products, such as essential oils for mite control. Even if propolis occurs in the beehive and may not be considered as a contaminant, if used as an acaricide, investigations on its potential use against Varroa mites are lacking. This is despite the fact that propolis showed biocidal activities against a range of microbes, parasites and ailments. One of the aims of this work is, therefore, to investigate the varroacidal actions of propolis.

1.2 *Galleria mellonella* as pest of the honeybees

The wax moth belongs to the subfamily Galleriinae of the family Pyralidae in which the females characteristically lay their eggs in beehives. This subfamily consists of two species known to be pests of the beehive, the greater wax moth *Galleria mellonella* and the lesser wax moth *Achroia grisella*. Both of these species have the same type of scavenging habits, but the lesser wax moth does not cause much damage, and hence is not a serious problem of beekeeping (Charrière and Imdorf 1997). Attention will, therefore, be given to the greater wax moth.

Galleria mellonella can be useful for the beekeeper in some aspects since it could recycle combs of colonies that die in the wild as well as the beeswax combs of the beekeeper. This moth can be reared purposefully as fish bait, animal feed, for scientific research, and it is a model system in insect physiology (Caron 1992). Regardless of its desirable uses in different fields, the

wax moth is seen as a honeybee pest by the beekeeper. Normally, the wax moth attacks only abandoned beehives, or active ones in which the bee colony has been weakened, e.g., as a result of disease or starvation. The beekeeper is more likely to see the adult moth, but it is the larval or caterpillar (worm) stage that causes damage to wax comb (Fig. 1.2 a and b). Wax moths fly mainly at night and rest in dark places during day time. They have acute sensory capability to find and exploit beeswax. Wax moths do damage during their larval stages, destroying combs and honey, but adults do not feed since they possess atrophied mouth parts (Charrière and Imdorf 1997). A female starts laying eggs 4 to 10 days after hatching (Shimanuki 1981) and produces 300 - 600 eggs in her lifetime, usually laid in batches of 50 – 150. These eggs are laid in cracks between hive parts, in dark and hidden places (Morse 1978). Wax moth eggs hatch to the larval stage in 5 to 8 days and the newly hatched larvae tunnel into the combs, leaving a complex of silken galleries behind. The larvae chew their way down to the midrib of the comb in order to be safe from patrolling adult honeybees, an important strategy of adaptation for their successful invasion of hives (Caron 1992). The tunnelling destroys the wax cells of the comb and causes leakage of honey due to puncturing of honey storage cells, making honey unmarketable. The larval stage feeds continuously on cocoon, faecal matter of the bee brood, debris, pollen, and wax (though indigestible), and it doubles its weight every day, under ideal conditions, for the first 10 days (Morse 1978). Wax moths prefer impurities in beeswax and, therefore, a comb used for brood rearing is at great risk. The larval and pupal development of a wax moth is aborted if it infests a colony containing only new and foundation combs, or combs used for honey (Morse 1978, Caron 1992, Charrière and Imdorf 1997).

Unlike most other parasites and pests of honeybees, the wax moth causes damage not only in a colony; it also causes destruction of stored combs. Dark and old combs used for brood rearing are the most difficult to store safely since they are full of cocoons and debris, and, hence, ideal to be infested by wax moths. Combs which are new or those used only for honey and stored in dry places have very little appeal to wax moth (Moosbeckhofer 1993). Wax moth larvae are most destructive to beeswax combs in storage, especially in areas that are dark, warm and poorly ventilated (Morse 1978).

In addition to the direct destructive impact caused by the larval stage, adults and larvae could also play roles in transmitting viral, bacterial, and fungal infections from infested to healthy individuals, facilitating demise of a diseased colony (Borchert 1966). Therefore, the control of *Galleria mellonella* in weak colonies and in honeycomb storehouses is very important. Different methods of control are available nowadays, but most of them are accompanied with one or more drawbacks, as will be demonstrated latter in chapter 6.

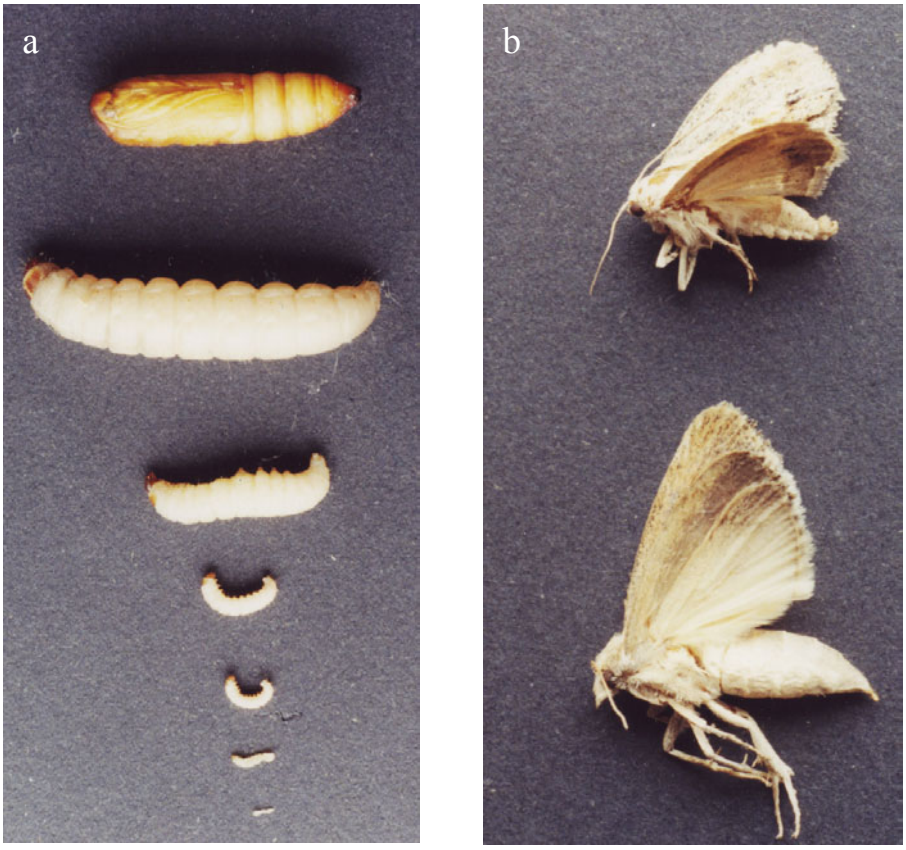

Fig. 1.2 Larvae and pupa (a), and a male (top) and a female (bottom) adults (b) of the greater wax moth *Galleria mellonella*

1.3 Propolis

Propolis (bee glue) is a resinous sticky gum collected by honeybees from various plants. Many plants have evolved mechanisms of protecting their leaves, flowers, fruits, buds, pollen, and prevent infection of wounds by producing a resinous substance with potent antimicrobial, anti-putrefaction, waterproof and heat-insulating properties (Münstedt and Zygmunt 2001). Resin oozes out after injury of a plant part in order to stop further sap loss and prevent infection of the wound, or it could be actively secreted as a protective covering of buds, to inhibit sprouting and subsequent death while frost (Crane 1990). Honeybees make use of the result of this long time evolution of plant secondary metabolism to protect their hives from infection (König and Dustmann 1988).

Honeybees collect resin from cracks in the bark of trees, leafs, boughs, and leaf buds, and masticate it by adding salivary enzymes. The masticated gum is then mixed with beeswax and/or other foreign materials, based on need for further use (Ghisalberti 1979, Marcucci 1995). It has been noted by Marcucci (1995) that the compounds in propolis originate from three sources:

plant resins collected by bees, secreted substances from bee metabolism (wax and salivary enzymes), and foreign materials which are introduced during propolis elaboration. The relative composition of the three different components varies based on the geographical location of the hive, vegetation composition, bee species, and availability of propolis source plants (Meyer 1956, Johnson et al. 1994, Burdock 1998). The more the available plant resin the less the proportion of wax and foreign materials added to make propolis, and vice versa. In seasons and locations where propolis source plants are scarce colonies suffer from propolis shortage and bees were observed collecting "propolis substituents", like asphalt, paint, and mineral oils (König 1985). These propolis substituents are then mixed with the available resin and used in the beehive. Even under favourable conditions of propolis collection, where there is no shortage of plant resin, the relative proportion of wax added to make propolis is dependent on the purpose for which it is to be used (Meyer 1956, Johnson et al. 1994). Propolis used to repair honey combs is often supplemented with large quantities of wax to give a firmer composition, whilst propolis applied as a thin layer on the internal wall of the hive contains very little or no wax, since wax has no antimicrobial effects (Ghisalberti 1979). The colour of propolis varies from yellow green to dark brown depending on the source plant species and its age. Its consistency is highly affected by temperature: being sticky and pliable above 30 °C, hard and unbreakable at about 15 °C, and brittle and easily pulverizable at a temperature less than 5 °C, especially when frozen (Hausen et al. 1987). The melting point of propolis lies on average between 60 and 70 °C, and it could even go up above 100 °C (Neunaber 1995).

1.3.1 Botanical origin

The fact that bees collect plant resins to prepare propolis was confirmed for the first time by Rösch (1927). Though the botanical origin of propolis was then generally accepted, it was not clear which plant species were used as sources. The difficulty in the identification of the plant species used as propolis sources lay mainly in the fact that propolis collection is a rare event carried out by few bees specialized for this purpose, and also that it often takes place high up in the trees (Crane 1990). The identification of the source plants of propolis involved observation of plants which the propolis collectors visit and comparative chemical analysis of propolis and plant resins (Bankova et al. 1992). The comparative chemical analysis of propolis and resin exudates of trees suspected to be propolis sources (mainly poplar and birch) started at the beginning of the 1970's, and similar chemical compositions between propolis and the corresponding resins were confirmed (Lavie 1976, Popravko 1978).

Nowadays it is commonly accepted and chemically demonstrated that the bud exudates of *Populus spp*. and their hybrids are the main sources of bee glue in temperate zones, such as in Europe (Tamas et al. 1979, Popravko and Sokolov 1980, Nagy et al. 1986, Greenaway et al. 1987), North America (Garcia-Viguera et al. 1993), the non tropical regions of Asia (Bankova et al. 1992), and New Zealand (Markham et al. 1996). In Russia, especially in the northern part, however, the main source of propolis is the birch *Betula verrucosa* (Popravko and Sokolov 1980). Apart from poplar and birch, other plant species, such as conifers (*Pinus spp*.), horse chestnuts *(Aesculus hippocastanu)*, *Prunus spp*. (almond, apricot, cherry, nectarine, peach, and plum trees), willow (*Salix spp*.), alder (*Alnus spp*.), oak (*Quercus spp.*), and hazel trees (*Corylus spp*.) play minor roles as propolis sources in temperate regions (Ghisalberti 1979). In tropical regions there are no poplars and birches and bees have to find propolis source plants (Bankova et al. 2000). The propolis source plants in tropical regions are highly variable due to the immense biodiversity of the flora. For this reason different plant species have been confirmed as propolis sources in various tropical countries. Some of the propolis source plant species, confirmed by observation of the flight activities of propolis collectors and by comparative phytochemical analysis include: *Cistus spp*. (Martos et al. 1997); *Ambrosia deltoidea* (Wollenweber and Buchmann 1997); *Clusia major* and *Clusia minor* (Guttiferae) (Tomas-Barberan et al. 1993); *Acacia spp.*, *Eucalyptus spp.,* and *Xanthorrhoea spp.* (Ghisalberti et al. 1978); *Araucaria spp.* (Bankova et al. 1996, Bankova et al. 1999), *Baccharis spp.* (Banskota et al. 1998, Marcucci et al. 1998, Bankova et al. 1999). Most of the data about the propolis source plants in the tropics relate to Australia, Brazil, and some other South American countries. The plant origin of propolis in most African, tropical Asian, and some South American countries is not yet known.

1.3.2 Collection

Propolis is collected by worker bees that are older than 15 days and specialized for this purpose (Bogdanov 1999). These bees are usually older than those that build comb and cap honey cells, and their wax glands are atrophied (Ghisalberti 1979). Since propolis is hard and difficult to handle at lower temperatures, bees usually collect it in the late afternoon of warm seasons of the year when it is relatively flexible, though very sticky. Bees were observed collecting propolis starting from spring up to early autumn in warmer regions of Europe such as Italy. In most other parts of Europe and the temperate zone in general, the high time for propolis collection was confirmed to be late summer and early autumn, and propolis collection is considered to be a preparation for overwintering (Bogdanov 1999). Due to the sticky nature of propolis, it is not a simple task for the bees to collect it, but the further processing and use in the

beehive becomes relatively simpler due to the addition and mixing with salivary gland secretions and wax (Droege 1989). A propolis collector bee may collect and carry up to 10 mg propolis (Fig. 1.3 a and b). Depending on the bee race and the geographical location of the hive, a colony in Europe is able to collect 50 to 150 g propolis per year; the Caucasian bees, however, can collect up to 1000 g (Bogdanov 1999). It is possible, however, to provoke the bees to go for more propolis collection. At present, one of the best methods used for commercial production of propolis is to place a plastic mat with mesh size not more than 4 mm under the top cover inside the hive. Other methods of provoking the bees for more propolisation include sending a drought through a hole in the hive (Bogdanov 1999), placing a mouse dummy, and sending a strong electromagnetic field over the beehive (Horn 1981).

Fig 1.3 A propolis collector bee with propolis load on the corbicula (a), and while "stealing" propolis from a chunk of it left on the hive by the beekeeper (b).

1.3.3 Uses in the beehive

Bees make use of the two important features of propolis in the beehive: mechanical and biological. The mechanical uses of propolis include its application as a thin layer on the inner wall of the hive or other cavities they inhabit. This may prevent loss of moisture in dry seasons (Baier 1969, Möbus 1972) and its catastrophic influx following heavy rainy seasons (Münstedt and Zygmunt 2001), enabling the bee community to keep the hive interior at a desirable moisture level. The presence of propolis as a thin layer also acts as a varnish, smoothing out the internal wall, making it more slippery, and enabling the honeybees to blow off invading ants (Münstedt and Zygmunt 2001). Propolis is also used to block holes and cracks less than 5 mm in diameter (crevices of diameter more than this could be filled-up with wax) (Droege 1989), to repair combs, to strengthen the thin borders of the comb, and for making the entrance of the hive

weathertight or easier to defend. This latter mechanical use of propolis might have led to the origin of its name from two words in ancient Greece: *pro* (for, in front, in defence) and *polis* (city, community), to refer to the substance for or in defence of the hive, analogous to walls or fences built around towns/cities to protect them from enemy attack in ancient times. The cape honeybee *Apis mellifera capensis* has been observed using propolis for encapsulation (imprisoning) of the parasitic small hive beetle (SHB) *Aethina tumida*, which could not be killed because of its hard exoskeleton and defensive behaviour, trapping and starving it to death (Neumann et al. 2001). In addition to the mechanical use, the presence of propolis in the beehive also has biological roles; it is used to embalm dead intruders which the bees have killed but could not transport out of the hive, thereby containing putrefaction. It is therefore responsible for the lower incidence of bacteria and moulds within the hive than in the atmosphere outside (Ghisalberti 1979). Propolis is applied as a thin layer on the inner wall of the comb cells before the queen lays eggs, probably to protect the brood from microbial infection (Droege 1989). The presence of propolis at the hive entrance plays not only a mechanical role, but also a biological one in that it acts as a repellent or simply reduces the attention of potential intruders, perhaps disguising the hive chemically as a part of an uninteresting plant (Münstedt and Zygmunt 2001). Propolis also acts as an inhibitor of seed germination and bud sprouting in the beehive, thereby preventing invasion of the hive by plant life (Ghisalberti 1979).

1.3.4 Chemical composition

The chemical make-up of propolis is mainly determined by the resin exudates of plants; the metabolic products of bees i.e. salivary enzymes and wax added to it, as well as foreign materials incorporated during refining play minor roles. Most plant resin components are incorporated into propolis without alterations, but it is likely that some of the components are subject to enzymatic changes by the bees' saliva during the collection or addition of the exudates to bees' wax to make propolis (Greenaway et al. 1990, Burdock 1998). The enzymatic changes may include chopping the carbohydrate components of flavonoid glycones with glucose oxidase to convert them to flavonoid aglycones (Greenaway et al. 1987). The specific chemical composition of propolis is highly influenced by the geographic location of the collection site and the collecting bee species. The largest group of compounds reported in propolis are flavonoid pigments which are ubiquitous in the plant kingdom. There are in general more than 200 hitherto identified compounds that belong to: amino acids, aliphatic acids and their esters, aromatic acids and their esters, alcohols, aldehydes, chalcones, dehydrochalcones, flavanones, flavones, hydrocarbons, ketones, terpenoids and other compounds (Marcucci 1995, Bankova et al. 2000).

Of these compounds, the flavonoids have been the most investigated and were shown to be responsible for the different biological activities ascribed to propolis.

Regardless of the high variation in the specific chemical make-up of propolis collected from different geographic locations and by various bee species or subspecies, its general chemical make-up, under favourable propolis collecting conditions, remains almost the same. It is generally composed of about 50% resin and vegetable balsam (components extractable in ethanol), 30% wax, 10% essential oils, 5% pollen and 5% various other substances including organic debris (Ghislaberti 1979).

1.4 Calorimetry in biological investigations

All physical, physicochemical, chemical and biochemical reactions are associated with the production or consumption of heat and, therefore, with the flow of heat between the system and its surrounding. Calorimeters are instruments used to measure such heat and heat flow rates. Thermodynamically defined, processes that liberate energy such as catabolic cellular reactions are called exothermic/exergonic, and those that absorb energy, such as anabolic/biosynthetic cellular reactions are called endothermic/endergonic. During the catabolic degradation of a substrate into its intermediate or end products, part of the liberated enthalpy is conserved in the production of ATP, and the rest is evolved as heat (Q_{cat}). Part of the energy stored in ATP, which is formed during catabolic reactions, is consumed by biosynthetic (anabolic) reactions, and the rest is given off as heat (Q_{anab}). The net heat production of life process/metabolism (Q_{met}) that one can measure calorimetrically is, thus, the sum of Q_{cat} and Q_{anab}. The calorimetric monitoring of this heat flow between a system and its surrounding could involve analytical calorimetry, whereby the qualitative question whether heat is produced/absorbed or not is answered, or quantitative calorimetry, that measures the amount of heat released/absorbed (Lamprecht et al. 1991).

1.4.1 Types of calorimeters

Different types of calorimeters are in use in various fields of science nowadays, with the classification being done by the use of a combination of several criteria/working principles of the calorimeters. The reader interested in the criteria used, and classification of calorimeters into groups is referred to Hemminger and Sarge (1998).

Calorimeters are commonly divided into batch and flow instruments based on the position of the reaction vessel/fermenter (Lamprecht 1983). Batch calorimeters are those types in which a closed vessel within the calorimeter contains all necessary ingredients for the reaction

and perhaps auxiliary equipments for initiating the reaction, stirring, mixing, illuminating etc. In flow calorimeters, however, the reaction occurs in a separate fermenter or vessel, outside the calorimeter, and only a part of the solution is pumped through the flow spiral of the calorimeter, where the heat production rate is measured. The calorimetric experiments with insects and mites in this thesis work were carried out with batch microcalorimeters, whereas all microbiological experiments were done with a flow microcalorimeter.

All the batch and flow microcalorimeters used in this thesis work are based on the heat exchange/conduction principle, whereby the heat produced in the calorimetric vessel is conducted to the surrounding heat sink (isothermal jacket) with an enormous heat capacity, to maintain the temperature of the calorimetric vessel constant. Such calorimeters are still referred by most calorimetrists as isothermal calorimeters, whereby the temperature of the surrounding (isothermal jacket) and the calorimetric vessel remains constant. The assumption here is that since the heat produced is transferred to the heat sink immediately, the temperature of the reaction vessel and the surrounding remains constant. However, for heat to be measured, it has to flow from a higher to a lower temperature gradient across the thermopile wall, thus, an ideally isothermal state can not be achieved (Hemminger and Sarge 1998). Therefore, in a strict sense, isothermal calorimeters are actually isoperibolic, whereby the surrounding has a constant temperature, with temperature of the measuring system possibly varying from it.

The construction principle of calorimeters could be a *single measuring system* or a *twin/differential measuring system*. The heat conduction types of calorimeters are generally constructed on the twin/differential principle, and are essential when high precision is required for slow process microcalorimetry (Kemp 1998). The two vessels of the twin setup (the reference and reaction vessels) are arranged as perfect twins with the detection units being in opposition, in order to give a differential signal. Thus, extraneous disturbances are cancelled giving long-term stability and precise results.

Isoperibolic calorimeters are among the most important types of calorimeters used in the investigations of living systems without interfering with their physiological demand. Such calorimeters are important in biological investigations because most biological reactions have a narrow optimum temperature range which can not be maintained by the other calorimetric types. The calorimeters used in this thesis work are categorized as *isoperibolic differential heat conduction microcalorimeters*; their working principle and interpretation of signals shall be considered in the next section.

1.4.2 Isoperibolic heat conduction microcalorimeters

Isoperibolic heat conduction microcalorimeters involve the transfer of heat produced in the reaction vessel to the surrounding heat sink (isothermal jacket), due to temperature difference across a thermopile wall (sensor of heat flow) placed between the vessel and the surrounding (Wadsö 2002).

The rate of heat evolution in the reaction vessel (rate of heat change) is P = dQ/dt, and is measured in units of watt (W). This rate of heat change is sometimes called "thermal power", but this remains disputed (Gnaiger 1993). Part of the heat evolved in the reaction vessel is exchanged with the surrounding, and a part of it is contained in the sample and in the reaction vessel that contains it. The sum of both is equal to the rate of heat change

$$P = dQ/dt = \Phi + C*dT/dt \qquad (1)$$

where Φ is the heat flow (rate of heat exchange) between the reaction vessel and the surrounding. The term C*dT/dt represents the rate of heat accumulation in the reaction vessel. C is the heat capacity of the sample and the reaction vessel system (including part of the measuring sensors), and dT/dt is the rate of change of the temperature of the reaction vessel. The heat flow rate from the reaction vessel to the surrounding, Φ, is directly proportional to the temperature difference between the reaction vessel and the surrounding

$$\Phi = K(T_{sample} - T_{surrounding}) \qquad (2)$$

where K is the coefficient of thermal conductivity between the reaction vessel and the surrounding. By combining equations 1 and 2 above, the rate of heat change, P, in the sample is related to the temperature in a calorimetric system as given by equation (3)

$$P = K(T_{sample} - T_{surrounding}) + C*dT_{sample}/dt \qquad (3)$$

The ratio between the total heat capacity of the measuring system C, and the heat exchange coefficient between the sample and the surrounding is the time constant of the calorimeter, τ, and is very important to consider for reaction kinetics where it is necessary to observe the beginning and/or end of a reaction.

$$\tau = C/K \qquad (4)$$

The total quantity of heat, Q, evolved in the reaction vessel, during a given experimental period can be determined by integrating equation 3:

$$Q = \int P = \int_{start}^{end} \Phi dt + C\Delta T \qquad (5)$$

The heat conduction calorimeters measure the heat flow, Φ, between the reaction vessel and the surrounding, thus the second term in equation 3 becoming a dynamic correction factor. For slow reactions, like the biological systems investigated in the present case, and for close to perfect heat conduction calorimeters, the $C*dT_{sample}/dt$ is insignificant and equation 3 becomes

$$P = \Phi \quad (6)$$

In (nearly) perfect heat conduction calorimeters the rate of temperature change in the sample and reaction vessel, ΔT in equation 5, is zero and integration of measured heat flow Φ gives the heat quantity, Q.

1.4.3 Calibration of calorimeters

Calorimeters can be calibrated by different means, such as by using the transition enthalpies of known reference materials, specific heat capacity, or direct electrical calibrations (Haines et al. 1998), or heat of chemical reactions (e.g. hydrolysis, and neutralization) of selected compounds (Briggner and Wadsö 1991, Kemp 1998, Beezer et al. 2001, O'Neill et al. 2003). Except for two of the Calvet calorimeters, where external resistors were employed, all calorimeters used in this study have built-in calibration heaters of known resistance. For this reason, and also since electrical calibration is a convenient method that can be done routinely as often as possible, calibration of all calorimeters was carried out electrically. For those calorimeters with no built-in calibration resistor, electrical calibration was done by passing precisely controlled electrical current by means of a constant current supplier (Electro Automatic EA, Viersen, Germany), through a calibration heater with a resistance of 124 Ω.

Electrical calibration can be done by passing a current, I (A) of known quantity through a resistor and recording the calorimetric output/thermopile potential, U (V) which is directly proportional to the temperature difference between the reaction vessel and the surrounding heat sink. The power input P is represented by

$$P = I^2 R \quad (7)$$

where I is the current input (A) and R is the resistance (Ω) of the calibration heater. The sensitivity of the calorimeter S (V/W) is the ratio between the thermopile potential U (V) and the power input of electrical energy P (W). For nearly perfect heat conduction calorimeters the power input P is the same as the heat flow rate from the reaction vessel to the surrounding (Φ) as displayed in equation 6. Thus,

$$S = U/\Phi \quad (8)$$

Calibration has to be carried out under conditions as close as possible to those of the sample experimental run. The flow calorimeters, especially, have to be calibrated (electrically) during pumping the sterile growth medium (in case of microbial investigations).

1.4.4 Advantages and disadvantages of calorimetry in biological investigations

Biological calorimetry is a general, non-specific method that measures the net enthalpy change that results from the complex metabolic reactions of living systems. The advantages and drawbacks of biological calorimetry both lie in the fact that measurement of heat flow is unspecific (Lamprecht 1983). The advantage of its non-specificity is that it monitors all heat producing reactions and, hence, can detect unexpected life processes that could be overlooked by other more specific methods (Lamprecht 1983, Wadsö 2002). The non-specific calorimetric signal from a complex biological reaction is unfortunately difficult to interpret at a molecular level in the absence of more specific analytical information. This difficulty can, however, be solved by equipping the calorimetric vessel (or the flow line in case of flow microcalorimeters) with specific analytical sensors such as electrodes and spectrophotometers, making the setup a very powerful analytical instrument for the interpretation of complex biological reactions (Johansen and Wadsö 1999).

Advantages of the calorimetric method compared to other techniques include its non-invasive nature - measuring heat production without interfering with the organism, and no need of clear solution – unlike spectrophotometric methods. In addition to this, the calorimetric method has a higher sensitivity compared to most standard methods, such as in the investigation of sublethal effects of toxicants on the metamorphosis of insects.

The position of the reaction vessel, deep in the calorimetric chamber, makes it difficult to mix the contents, and to supply essential materials such as oxygen in batch calorimeters, because stirring and/or pumping can introduce artefacts in the calorimetric signal (Lamprecht 1983). These problems can be minimized by the use of flow calorimeters, but the problem of exhaustion of oxygen in the flow line still persists, especially at higher cell densities and hence interpretation of results has to be done with caution and in combination with signals from oxygen sensors incorporated in the flow line.

1.5 Objectives and structure of the thesis

Chapter 1: As aforementioned at the beginning of this chapter, it is important to give general introductions about Varroa mites and varroosis, wax moths, propolis, and calorimetry. Thus, in this chapter the biology of *Varroa destructor* mites, the extent of problem of varroosis, possible means of coexistence of some resistant honeybee species/subspecies with the mite, by controlling the population size below a certain threshold are demonstrated. A brief insight into the biology and infestivity of *Galleria mellonella* is made in order to show the extent of the problem. In addition, this chapter also gives clues as to the what-about of propolis, its chemical

make-up, botanical origin, and collection and use by the honeybees. The last part of this chapter deals with the important technique, calorimetry, used in most investigations of this thesis research. The calorimetric topic demonstrates the working principles, sensitivities compared to other methods, reliability, and calibration and standardization of calorimeters.

Chapter 2: It is still highly controversial among bee researchers and beekeepers, as to whether the energy and nutritional demand of *Varroa destructor* mites and, thus, the amount of hemolymph they suck from their host (mainly brood), is by itself responsible for the weakening and consequential death of honeybees. Most of the hitherto evaluations of the energetic and nutritional demand of Varroa mites are more of speculations rather than experimental proofs. Several researchers demonstrated that *Varroa destructor* mites transmit viral infections. The transmitted viruses were considered to play primary roles in killing bees, whilst the mites playing a secondary one, or just simple vectors with no much impact on the bees. This confusion and debate about the role of *Varroa destructor* arises mainly due to undermining the amount of hemolymph they suck from brood. Thus, this chapter is devoted to the experimental and computational proofs of the amount of hemolymph mites suck, and their energy demand from a capped brood. Calorimetric methods are used in the investigations of the energy demand of Varroa mites and brood during the capped developmental stage. The amount of hemolymph the mites suck during brood development is evaluated by starving the mites, incubating them in the absence of their host, and measuring the weight loss. The logic behind measuring weight loss of starving mites is that had it not been for the absence of their host, they would have sucked an amount of hemolymph equal to the lost weight and maintained their weight constant. The length of time spent by each individual (mother and offspring) mite in a capped brood will be taken into consideration for the computation of mites' energy and nutritional demand.

Chapter 3: Varroa destructor has become a global problem of the beekeeping industry based on *A. mellifera*. In order to stop the weakening and consequential death of colonies, beekeepers are treating them with acaricides. The use of acaricides, however, is associated with residue problems in bee products and mites resistant to currently used acaricides have already emerged. These problems provide incentives to search for new and potential acaricides which are free from the mentioned problems. Natural products are becoming the subject of such investigations. This chapter deals with the *in vitro* investigations of the acaricidal action of propolis. The Varroa weakening and varroacidal actions of various concentrations of propolis extracted in 70% or 40% ethanol are evaluated with different lengths of contact times. The screening for optimum concentration and contact time may help in the future development of treatment method *in vivo*. Evaluation of the effects of various concentrations and treatment times

are carried out by counting the number of dead/inactivated mites at different time intervals, and through calorimetric monitoring of the heat production rates of mites before and after treatment with propolis.

Chapter 4: The use of high temperature treatment (e.g. 44 °C) to differentially kill the infesting mites in a capped brood was found out to be effective with little impact on the latter. One of the drawbacks of high temperature treatment is the length of exposure time, leading to brood/bee death. It is, therefore, desirable to develop a method of shortening the treatment period. One of such methods would be the exploitation of the synergistic effect of lower to moderately concentrated acaricides and high temperature treatments. This chapter concentrates on the calorimetric and respirometric investigations of the effect of temperature on the antivarroa actions of propolis. Investigations are carried out on mites collected from drone brood, worker brood, and adult workers separately, since they may have different responses. It is usually recommended that calorimetric results have to be supported by other data, to concretely explain the changes that take place in the experimental organism. The calorimetric results at different temperature setups are compared with those of manometric experimental results.

Chapter 5: Propolis samples from different geographic origins were shown to be highly variable in chemical composition. It is not clear whether this variation in chemical composition affects the antivarroa action of propolis or not. Comparisons of the antivarroa action of propolis of different geographic origins are made in this chapter. Apart from samples of different geographic origins, the antivarroa actions of propolis samples from the same location, and even from the same apiary but different beehives are compared. The species/subspecies of bees that collected propolis are considered whilst comparing the antivarroa actions of different samples. The differences in the antivarroa actions of various extracts of the same sample are also made.

Chapter 6: Apart from *Varroa destructor,* the beehive harbours several parasites and pests that cause enormous loss, and have to be controlled. One of such pests is the greater wax moth *Galleria mellonella*. Beekeepers treat their colonies with insecticides to save them from death, but insecticides used against wax moths cause residues in bee products, and may irritate bees and beekeepers. One of the best solutions to the problems associated with synthetic insecticides is the use of natural products. This chapter deals with the *in vitro* investigation of the effects of propolis on the metabolism and development of the different developmental stages of *Galleria mellonella*. In a first group of experiments the effect of propolis on the heat production rates of the different larval stages are investigated to evaluate the change of sensitivity to propolis treatment with changing larval instars. Since the treatment with lethal doses of propolis does not make sense for calorimetric experiments, most of the calorimetric investigations are

carried out with sub-lethal doses. Apart from the reduction of heat production rate of an organism, by weakening it, some plant secondary metabolites were observed to play the roles of insect growth regulators, either by facilitating or retarding larval and/or pupal development. The second group of experiments, thus, concentrates on the effect of sublethal doses of propolis on pupal metamorphosis. This is achieved by treating the last larval instar of Galleria *mellonella* with propolis, and calorimetrically monitoring the events of metamorphosis up to adult emergence. Different events and parameters of metamorphosis, like the strength of endothermic trough and exothermic peaks during ecdysis, length of the metamorphotic period, the basic metamorphotic heat production rate, rate of pupal reserve food utilization, etc., are evaluated.

Chapter 7: Propolis is claimed to be a multifaceted drug against various types of bacterial and fungal infections. Several investigations were carried out in different laboratories to proof its potential, but most of them have one or more limitations. Insight into possible solutions of the existing limitations of propolis antimicrobial research, new queries and problems are dealt with in this chapter. The majority of hitherto investigations were based on only one type of extract, mainly the ethanol-extracted propolis, and experiments with other types of extracts are rare. The use of aqueous solutions of propolis, however, could be desirable under certain circumstances, especially for human medicinal use. Therefore, the antibacterial and antifungal activities of three types of extracts of propolis, namely the ethanol-extracted propolis (EEP), water-extracted propolis (WEP), and Propolis volatiles (PV) are investigated and compared in this chapter. In addition, comparison of the antimicrobial activities within, and between different extracts is made in relation to some physico-chemical parameters, and the yield of propolis extraction.

Almost all of the hitherto antimicrobial investigations of propolis were carried out using the Petridish bioassay method which is actually a highly constrained method for the investigation of the mechanisms of action of antimicrobial agents, especially the hydrophobic ones. In this chapter, the mechanisms of action of propolis are investigated by means of flow microcalorimetry coupled with polarographic oxygen sensors. Results from the flow microcalorimetric investigations are compared with those obtained from the Petridish bioassay methods.

Up to now most propolis samples used by researchers were usually obtained from a certain geographic region. Comparisons of samples from regions of completely different climatic zones are rare. Hence, this chapter deals with various extracts of propolis samples obtained from geographic regions of different climatic zones and vegetation compositions, in order to observe the effect of geographic origin on the activity of one or more of the extracts.

Chapter 8: A general discussion of the extent of problems of honeybee parasites and pests, currently existing solutions to these problems and their drawbacks are given in this chapter in English and German languages. In addition, the findings of the different chapters of this thesis work, and their potential *in vivo* applications in the control of such parasites and pests are dealt with.

Chapter 9: This chapter gives short summaries of the thesis in German and English languages.

Chapter 10: Here the emphasis is on planned future work, and what has to be done before propolis is to be used in the control of honeybee parasites and pests.

Chapter 11: Contains the list of reference materials used during the research phase and while writing the thesis.

Chapter 12: Is a compilation of appendix, personal data, a list of my own publications, and declaration.