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Calorimetrical and biochemical investigations on the influence of the parasitic mite *Varroa destructor* on the development of honeybee brood

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Abstract

The parasitic mite *Varroa destructor* is a serious pest to the western honeybee *Apis mellifera*. Here, we investigated the impact of mite infestation on the energy content, energy density, hemolymph volume and hemolymph protein concentration of drone and worker pupae. Mite infestation had a significant impact on the energy content of worker pupae at the end of metamorphosis, with an energy content of highly infested pupae 15% lower than non-parasitized pupae. The energy content of infested and uninfested drone pupae did not differ significantly. The energy density of drones and workers was not affected by mite infestation. Drones had a higher energy density compared to workers and lost more energy during metamorphosis. The heat production rates of drone pupae did not change due to mite parasitation. The total hemolymph volume of worker bees was significantly reduced in infested pupae, whereas the mites did not influence the hemolymph protein concentration. Although drones generally were less affected through mite infestation than workers, our results reveal a clear energetic impact of mite parasitation on honeybee pupae.

Keywords: *Varroa destructor*; Honeybee; Calorimetry; Energy content; Hemolymph volume

1. Introduction

In the western honeybee *Apis mellifera*, the most serious and for beekeepers economically desastrous bee pest are infestations of bee hives with the small parasitic mite *Varroa destructor* [1]. These mites enter brood cells of honeybees and feed on the hemolymph of the bee brood. Although the mites also parasitise adult bee workers who unwillingly transfer them to new brood combs or even other bee hives, the infestation of the developing bee brood is most damaging. Mites enter the brood cells shortly before pupation of the bees, when larvae undergo an ecdysis (molt) from larva to pupa. Shortly before this ecdysis, the cell is being sealed with a wax lid by nurse bees. Consequently, the mites are undisturbed and can not be easily detected by worker bees. Later on, the mites leave the cell after completion of the metamorphosis of the bee pupa. The young adult bee undergoes a second ecdysis from pupa to adult, and then the young adult nibbles away the wax lid of its cell and eventually hatches. Meanwhile, the mites have reproduced themselves on the bee pupa. Typically, one single female mite produces one son and four daughters. The male mite offspring mates with the female offspring, and the mites leave the cell together with the emerging young adult bee [2].

Parasitation with mites results in serious damage and malformation of the young bees [3]. Although the occurrence of crippled young bees is clearly associated with *Varroa* infestation, the causality between malformations and parasitation is not clear. Two hypotheses try to explain the connection between mite infestation and brood damage. The first one explains malformations as the product of secondary infections [4]. *Varroa* mites serve as a vector of these infections (e.g. deformed wing virus). As the correlation between malformation and the occurrence of diseases is unclear in some cases [5], a second hypothesis explains malformations and damage of the brood as a direct after-effect of the parasitation [6]. The mites consume large amounts of the bee's hemolymph and weakens the bee during a very sensitive phase of its development, the metamorphosis. As the metabolic rates of *Varroa* mites are very low, the amount of energy which is being robbed by the mites from the bee pupae must be negligible. Nevertheless, the amount of hemolymph sucked by

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the mites is remarkably high, and the metabolic efficiency of the mites must be low or the hemolymph is poor in nutrients [7]. However, up to now there are not enough data for a substantial support of the energy hypothesis. It was the goal of our study to close this gap.

We investigated the energetical impact of *Varroa* mite infestations on bee pupae. For this end, we determined the energy content of infested and non-infested bee pupae by means of combustion calorimetry. In addition to this, we measured the heat production rates of living infested and non-infested bee pupae in order to evaluate the direct effect of *Varroa* parasitation on the energy metabolism of bee brood. We determined the amount of hemolymph robbed by the mites and investigated the changes of protein concentration in the hemolymph.

2. Experimental

2.1. Honeybees

All experiments described in this study were carried out with brood organisms of the Western Honeybee *A. mellifera carnica*. The bees were kept in an apiary at the Institute for Biology/Zoology of the Free University of Berlin. *Varroa*-mites originated from natural infections in these hives. For determination of energy content and protein concentration, the bees within their brood combs were taken from the hives, killed and stored in a freezer at −18 °C. To determine heat production rates and hemolymph volume in living bee pupae, combs with bee brood were taken from the hives and stored in an incubator at 35 ± 0.5 °C. The age of different developmental stages of bee pupae was estimated by investigation of the color of the individuals according to [8]. In all experiments, the rate of infestation was carefully determined. Three categories of mite infestations were defined as: zero mite (non-infested), one to three mites (lightly-infested), four to six mites (heavily-infested).

2.2. Energy content

Frozen bee pupae were prepared from the brood combs. Age was determined as well as the number of mites in the cell. The pupae were weighted with a fine balance (type Sauter 414/3, Sauter, Ebingen, Germany) to the nearest 0.5 mg and then transferred to a small sample vial. The samples were lyophilized (GT2, Leybold-Heräus GmbH, Cologne, Germany) and weighed again for determination of the dry mass. The energy content of the samples was determined by means of a combustion calorimeter (self-construction after Phillipson [9]) connected to a chart recorder (BD 41, Kipp and Zonen, Delft, The Netherlands). Combustion took place in high-pressure oxygen atmosphere (25×10^5 Pa). The calorimeter was calibrated with pure benzoic acid. The sensivities for the three bombs used in our experiments were 93.8, 96.7 and 95.7 mV/J.

2.3. Heat production rates

For determination of heat production rates of living drone pupae, single capped cells (cells with closed wax lids) were prepared from a brood comb. As bee pupae are very delicate and sensitive, they were left inside their cells for measurements in order to prevent injuries. The number of mites and the developmental stage of the pupa were assessed through optical investigation as the wax walls of the cell were more or less transparent. An exact determination of the infestation rate and the age of the bee were made after the experiment. The heat production rates of bee pupae were measured by means of three isoperibolic batch-calorimeters (Calvet Ms 70 with a single twin unit, and Calvet No. 23063 with two twin units, both Setaram, Lyon, France and Biokalorimeter-B.C.P., Messgeräte Vertrieb, Munich, Germany) connected to chart recorders (Linseis L6532B, Selb, Germany and Kipp & Zonen BD41, Delft, The Netherlands). The calorimeters were calibrated electrically, and the sensitivities rendered 44.37 μV/mW (Biokalorimeter-B.C.P.); 62.63, 44.21 μV/mW (both Calvet No. 23063) and 51.15 μV/mW (Calvet Ms 70). All experiments were run at 35 °C.

The heat production rate was recorded for 60 min, the record of the power–time (*p–t*) curve was stopped and the pupa taken out of the measuring vessel. The pupa was then infected artificially with one, three or six mites, respectively. For this end, the cells were punctuated with a thin needle and the mites introduced to the cell. The hole was sealed after this, the cell containing pupa and mites again placed in the measuring vessel and inserted again into the calorimeter. The heat production rate of the newly infected pupa was recorded for further 60 min in order to investigate the direct effect of mites on the energy expenditure of the bees. For calculation of the specific heat production rate, body mass of the bee pupae was determined by means of a fine mechanical balance (Typ 414/13, Sauter, Ebingen, Germany) to the nearest 0.1 mg. The mean heat production rates of the bee pupae were evaluated by electronic integration (Digikon, Kontron, Munich) of the *p–t* curves and division by the experimental period.

2.4. Hemolymph volume

The hemolymph volume of drone and worker pupae was determined after [10], using amaranth red as a coloring agent. A defined amount of amaranth red, solved in Ringer's solution, was injected into the living pupae. After 30, 60 and 90 min, a defined amount of 10, 20 or 50 μl hemolymph, depending on the developmental stage, was withdrawn from the pupa with a micropipet. The hemolymph, now colored with amaranth red, was soluted in Ringer's solution and the optical density determined by means of a spectrophotometer (Titertek Multiskan plus, Type 311 A0, Eflab, Finland). For calculation of the hemolymph volume, the optical density of the samples was compared to a calibration curve with optical

density plotted against different concentrations of amaranth red.

2.5. Hemolymph protein concentration

We used a standard bicinchoninic protein assay kit (BCA-1 and B9643, Sigma, Deisenhofen, Germany) to determine the protein concentration in the hemolymph of worker and drone pupae. The pupae were frozen as described above, and their body mass obtained with a fine mechanical balance (Typ 414/13, Sauter, Ebingen, Germany) to the nearest 0.1 mg. Subsequently, the pupae were defrosted and hemolymph was withdrawn by means of a micro-capillary from the ventral area of the thorax, between the basis of the third leg pair. Three microliter of hemolymph were soluted in 87 μl aqua dest, 4 μl were taken from this solution and given to 1 ml of BCA-reagent in an Eppendorf-tube. After an incubation of 30 min at 60 °C in a water bath, the optical density of the solution was measured at 562 nm in a photometer (Titertek Multiskan plus, Type 311 A0, Eflab, Finland). To calculate the protein concentration, we calibrated the optical density of a standard bovine serum albumin solution at different concentrations using the same method.

2.6. Statistics

We used statistical software (Sigma Stat 2.03, SPSS Inc., Chicago, USA) for analysis of our data. After testing for normal distribution and variance, we applied the *t*-test for normally distributed data and the Mann–Whitney test for data where the normality test had failed. For comparison of three or more groups, we employed ANOVA for groups with

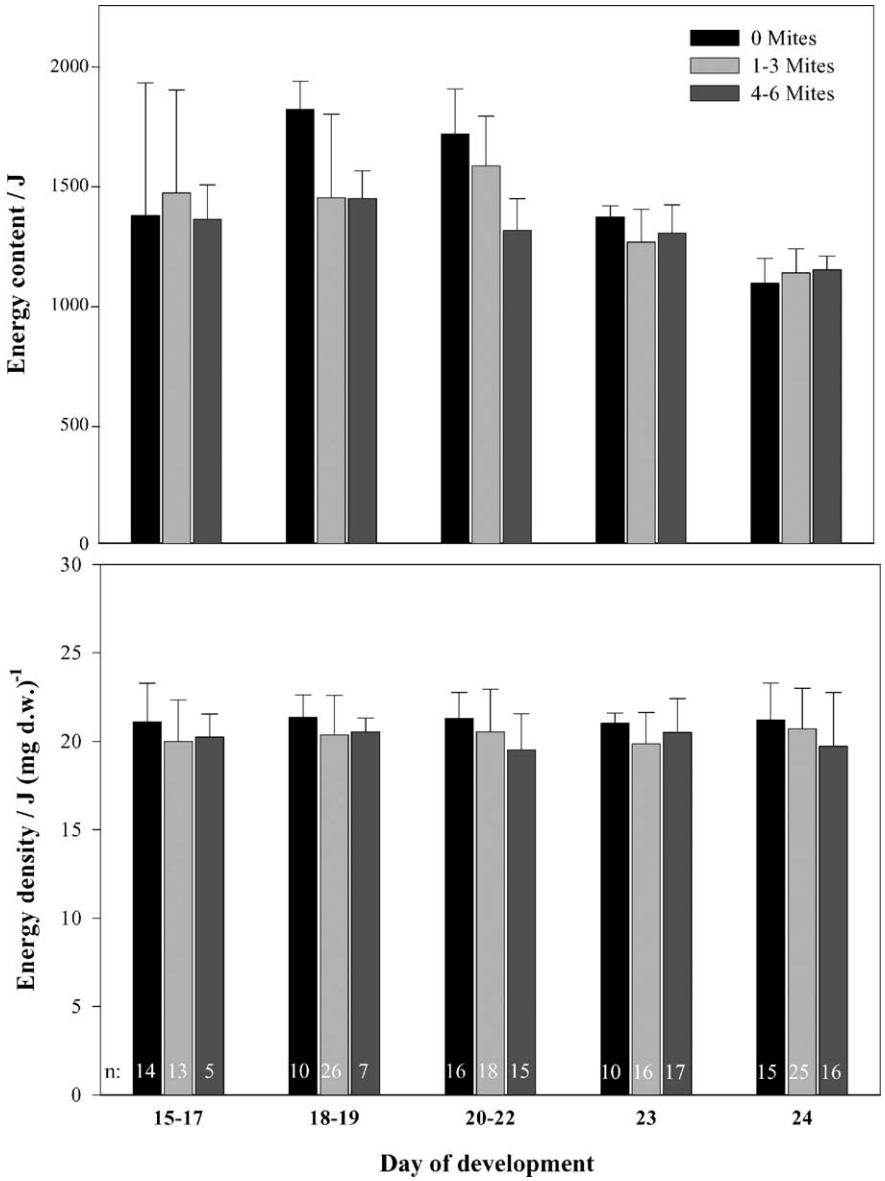

Fig. 1. Energy content and energy density of uninfested and infested drone pupae. White numbers on histogram bars indicate the number of experiments. Energy contents and energy densities were calculated from the same experiments.

the same variance. When significant differences appeared, we used the Tukey's test for further analysis. Accordingly, in groups which were neither normally distributed nor had the same variance, we applied the Kruskal–Wallis test and, if significant differences could be shown, Dunn's test for a detailed data analysis.

3. Results

3.1. Energy content

The energy content of uninfested drone and worker pupae (Figs. 1 and 2) decreased during metamorphosis from 1.82 ± 0.12 kJ ($n = 10$) in drones at days 18–19 of development to 1.09 ± 0.41 kJ ($n = 15$) at the end of the pupae phase (day 24). Worker pupae had a lower energy content than drones with 0.62 ± 0.05 kJ ($n = 11$) at the beginning (days 12–13) and 0.41 ± 0.03 kJ ($n = 10$) at the end of pupation (day 21). In relation to their energy content at the beginning of pupation, drones lost more energy than workers (40% of their energy content at beginning of pupation in drones and 34% in workers).

Mite parasitation had a clear impact on the energy content during pupation in workers, whereas the energy content of infested drone pupae did not differ significantly from uninfested ones. The energy content of workers infested with four to six mites was about 15% lower than that of uninfested pupae at day 21 and amounted to 0.35 ± 0.04 kJ ($n = 9$).

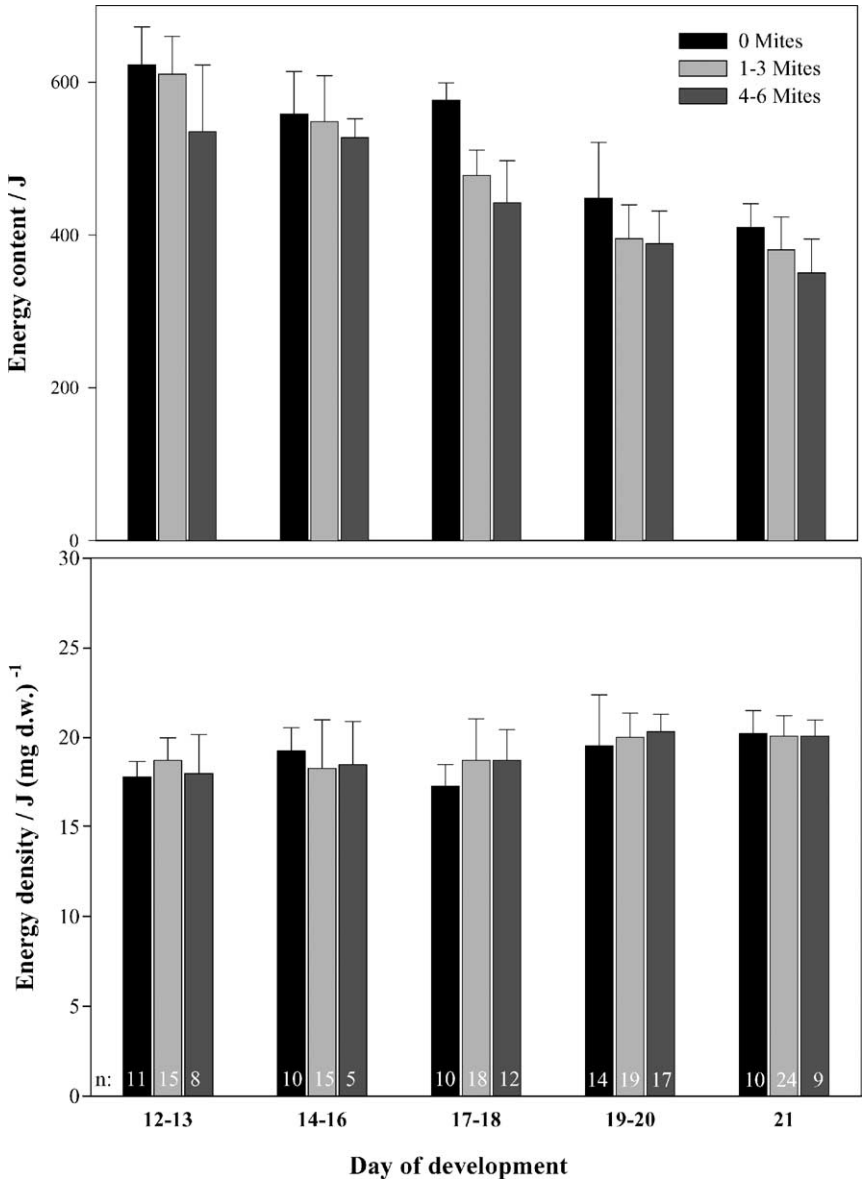

Fig. 2. Energy content and energy density of uninfested and infested worker pupae. White numbers on histogram bars indicate the number of experiments. Energy contents and energy densities were calculated from the same experiments.

The energy density increased significantly during metamorphosis of uninfested workers (17.8 ± 0.85 kJ/g d.w. at days 12–13 up to 20.21 ± 1.28 kJ/g at day 24; $P < 0.05$) and remained constant during the development of drone pupae. Uninfested drones had a higher energy density at the beginning of their metamorphosis than workers. The energy density of drone and worker pupae was unaffected by mite infestation, regardless of infestation levels.

3.2. Heat production rates

We determined the heat production rates of uninfested and of infested drone pupae before and after an artificial infection with additional one, three or six mites at two different developmental stages: days 18–19 and 20–22 (Fig. 3). Because of methodical reasons we were able to do a sufficient number of experiments only with these stages. Some additional experiments with earlier and later stages were performed, which were not evaluated statistically. Nevertheless, their results are in good accordance with those presented here. The infestation with mites which were already in the cells at the beginning of the experiments had no significant effect on the heat production rates of the pupae. With the exception of two experimental groups, the heat production rates did not change after more mites were additionally inserted to the cells. Only pupae at days 18–19, which were uninfested and then received a mite load of one mite and pupae from days 20 to 22, which were previously infested with one to three mites and then were infested with an additional mite, exhibited a significantly lower heat production rate after being additionally infested (days 18–19, zero mite, experimentally infested with one mite: from 2.9 ± 0.42 mW/g

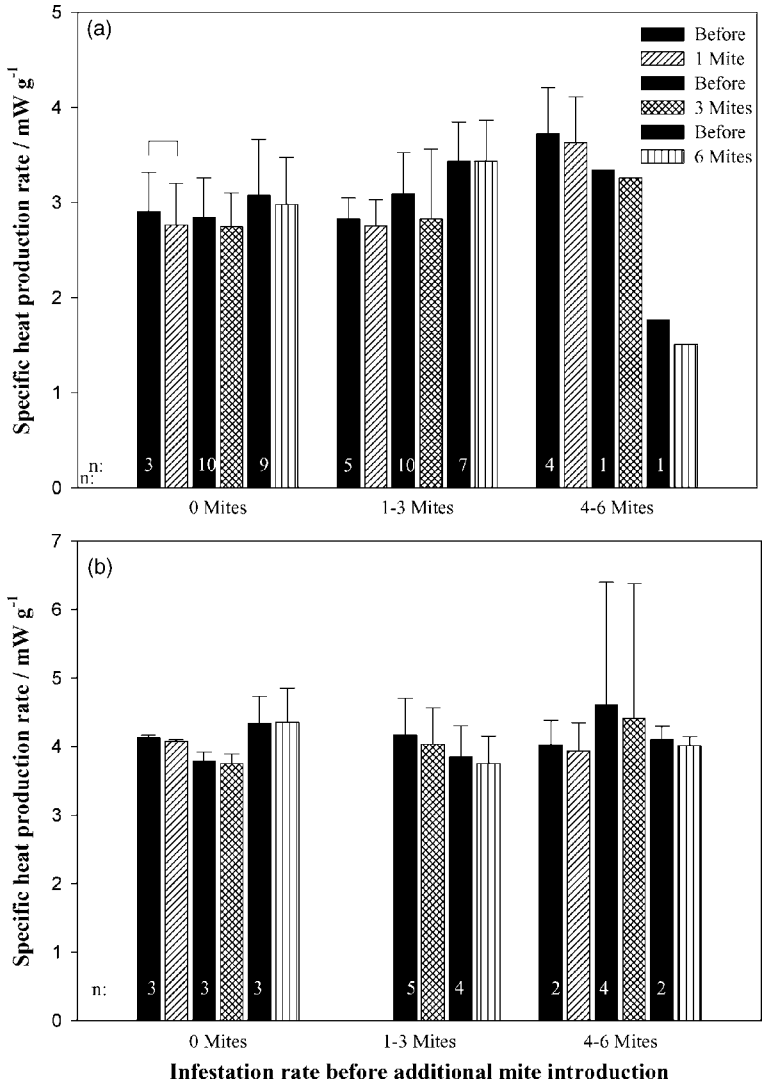

Fig. 3. Heat production rates of infested and uninfested drone pupae. Black bars indicate the heat production rates before experimental indroduction of mites to the cells. Right to each black bar shaded bars are shown for the corresponding heat production rates after mite introduction (for details see text). White numbers on histogram bars indicate the number of experiments. (a) Heat production rates of drone pupae after days 18–19; (b) heat production rates of drone pupae after days 20–22.

Table 1
Specific hemolymph volume (μl/(mg w/w)) of honeybee drone and worker pupae as function of development and mite infestation

Days of development	Zero mite (mean ± S.D.)	*n*	One to three mites (mean ± S.D.)	*n*	Four to six mites (mean ± S.D.)	*n*
Worker pupae						
12–13	0.222 ± 0.126	8	0.209 ± 0.073	6	–	
14–16	0.240 ± 0.114	8	0.118 ± 0.046	4	0.166 ± 0.062	5
17–18	0.581 ± 0.149	5	0.400 ± 0.104	5	–	
19–20	0.772 ± 0.165	7	0.712 ± 0.386	6	–	
21	0.523 ± 0.146	5	0.375 ± 0.171	9	–	
Drone pupae						
15–17	0.090 ± 0.044	6	0.085 ± 0.041	9	0.058 ± 0.001	3
18–19	0.097 ± 0.069	6	0.076 ± 0.046	8	0.064 ± 0.062	3
20–22	0.114 ± 0.039	7	0.046 ± 0.113	5	0.113 ± 0.047	7
23	0.157 ± 0.071	7	0.163 ± 0.171	5	0.171 ± 0.083	11
24	0.150 ± 0.063	7	0.104 ± 0.165	9	0.165 ± 0.057	11

n, number of experiments; S.D., standard deviation.

to 2.76 ± 0.44 mW/g; $P = 0.01$; days 20–22, infested with one to three mites, experimentally infested with one mite: from 4.17 ± 0.53 mW/g to 4.03 ± 0.54 mW/g; $P = 0.01$).

3.3. Hemolymph volume

Uninfested worker pupae have a similar hemolymph volume at the beginning of their metamorphosis as drones, but since drones have a higher total body mass at the beginning of pupation, the specific hemolymph volume is about twice as high in worker as in drone pupae (Table 1). In both drone and worker pupae, the specific as well as the total hemolymph volume increase during metamorphosis and decrease only during the last day of pupal development. In worker pupae, the specific hemolymph volume increases faster during development than in drones. Worker not only exhibited a higher specific hemolymph volume, but were also more affected by mite infestation. In drones, no clear effect of parasitation was visible whereas in workers, most developmental stages which were infested with one to three mites had a lower, but not significantly reduced specific hemolymph volume. Nevertheless, when comparing the total hemolymph volume one can find significant differences between healthy and infested worker bee pupae (days 20–22; $P = 0.036$).

3.4. Hemolymph protein concentration

The hemolymph protein concentration in both drones and workers decreased significantly from the first pupal stage on. Only in workers, the protein concentration was significantly lower in prepupae and then increased before pupation. Infestation with *Varroa* mites had no significant influence on protein concentrations of drone and worker pupae, except for young adults ready to hatch. In both drones and workers, young adults infested with one to three mites had a significantly higher protein concentration than uninfested ones (Table 2).

Table 2
Hemolymph protein concentration (mg/ml) of honeybee drone and worker pupae as function of development and mite infestation

Days of development	Zero mite (mean ± S.D.)	*n*	One to three mites (mean ± S.D.)	*n*	Four to six mites (mean ± S.D.)	*n*
Worker pupae						
9–11	101.5 ± 34.6	9	116.1 ± 35.0	6	–	
12–13	174.9 ± 28.5	17	169.8 ± 23.0	22	–	
14–16	152.9 ± 39.7	15	160.4 ± 34.5	19	–	
17–18	140.2 ± 44.8	13	129.3 ± 45.6	31	–	
19–20	64.1 ± 42.0	14	76.0 ± 35.5	29	89.6 ± 38.0	6
21	46.9 ± 9.8[a]	13	67.5 ± 19.2[a]	31	57.1 ± 14.4	10
Drone pupae						
11–14	152.7 ± 25.4	9	160.9 ± 27.6	9	159.5	1
15–17	151 ± 25.9	9	144.9 ± 26.9	13	154.8 ± 35.7	2
18–19	172.9 ± 27.1	18	153.3 ± 29.1	21	158.4 ± 28.5	6
20–22	134.3 ± 27.6	16	143.7 ± 26.0	27	148.0 ± 43.8	4
23	119.1 ± 26.7	12	120.5 ± 21.0	23	118.4 ± 14.0	19
24	70.4 ± 11.1[a]	10	111.6 ± 29.3[a]	25	88.9 ± 18.7	9

n, number of experiments; S.D., standard deviation.
[a] Significant difference between infested and uninfested group.

4. Discussion

The energy content of drone and worker bees decreased during metamorphosis. Worker pupae lost about 34% of their energy content they had at the beginning of pupation. Drones even consumed more energy (about 40% of their original energy content). Male wax moths (*Galleria mellonella*) lose a comparable 31% of their energy reserves during metamorphosis [11]. The energy density of bee pupae remains more or less constant; it is higher in drones, which have a higher body fat content. In *G. mellonella*, the energy density increases during the pupal phase, because more protein and carbohydrates are metabolized than fat, which remains as an energy depot of higher caloric value for the adult. In contrast honeybee pupae do not use up their energy depots selectively and have nearly no fat reserves for their adult life. Infestation with *Varroa* mites does not alter the energy density of bee pupae, which is a hint that mites feed unselectively on bee pupae (see also the discussion of hemolymph protein concentration), whereas it has a significant influence on the total energy content at the end of metamorphosis. Worker pupae infested with four to six mites have an energy content of about 15% below the normal value at the end of pupation. Unparasitized young worker bees ready to hatch had an average body mass of 107 mg, which is practically the weight of a emerged adult [12]. This means that bees have no energy reserves left after metamorphosis, as they do not need them for their life in a social colony were food is readily provisioned by nestmates. As a consequence, they have nearly no safety margin in their energy reserves during pupation when they can not take up any external food and are more vulnerable to starvation than other solitary insects like wax moths. Therefore, an energy loss of about 15% due to mite parasitation may have serious effects on the development of the bees.

Our study shows that the energy loss is not caused by elevated heat production rates. Mite infestation did not affect the energy metabolism of bee pupae. The energy loss can, therefore, be directly linked to the hemolymph robbing of the mites.

The specific hemolymph volume increased during metamorphosis in both drone and worker pupae up to the last day before the molt from pupa to adult. The relative amount of hemolymph compared to body mass increases due to the production of metabolic water in the pupae. Mite parasitation has no effect on drones were the results had a wide scatter, probably due to methodical reasons. In worker pupae, an effect on the hemolymph volume could be observed. The comparison of these results with values for the ratio of dry weight to wet weight (obtained from combustion calorimetric experiments) confirmed the results of an increased desiccation due to parasitation. Worker bee pupae infested with one to three mites lost about 35% of the hemolymph volume of unparasitized pupae of the same developmental stage.

The protein concentration of the hemolymph decreased in drones and workers throughout pupation, but remained unaffected by mite infestation. The protein concentration is lower in the prepupal stage than shortly after pupation. At this stage, a large amount of free protein can be found in the hemolymph, because the larval structures are being degraded. In the course of body reorganization the protein content of the hemolymph naturally decreases. Contrary to assumptions and findings published elsewhere [13], we could show that mites do not feed selectively on protein.

Our calorimetrical as well as biochemical results indicate strongly that bee pupae suffer from energy deprivation due to mite parasitation. Nevertheless, a direct proof that malformations of infested bees are caused mainly due to an energy loss and not to secondary infections can not be given. The most probable explanation for the occurrence of malformed bees in *Varroa*-infested bee hives up to now is a combination of both effects: the energy loss will at least weaken otherwise healthy bees and make them more vulnerable to secondary infections.

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