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Microcalorimetric toxicity investigation of propolis on *Tenebrio molitor* L. (Coleoptera: Tenebrionidae)

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Abstract

Toxicity of propolis (bee glue) against the three developmental stages (larvae, pupae, and adults) of the yellow meal worm *Tenebrio molitor* L. was investigated calorimetrically after dipping the animals for 60 s in different concentrations of propolis dissolved in 55% ethanol. The reduction in the heat production rate due to treatment with different concentrations of propolis displayed similar patterns in the case of larvae and pupae with the mean heat production rate being lowered by more than 90% due to treatment with propolis concentration of $\geq$7.5%. In addition the power–time (p–t) curves after treatment became smoother, the extent of smoothing being dependent on the concentration of propolis. Treatment of the adults, however, even with 10% propolis resulted in the reduction of the mean heat production rate by only 28%, with lower concentrations of propolis having no considerable impact.

Keywords: *Tenebrio molitor*; Propolis; Toxicity; Microcalorimetry; Narcosis

1. Introduction

Calorimetric methods are promising in the continuous monitoring of life activities since the latter are associated with the production of heat. In the field of insect physiology, apart from others, microcalorimetry has been applied in the investigation of insect growth and development [1–6] and the toxic effects of plant secondary metabolites as inducers of morphogenetic failures in pupae [7,8]. It has been pointed out [9] that terrestrial insects are the most frequently calorimetrically investigated small "calorimeter-sized" animals since they are easy to gather, breed, keep, handle and measure in dry vessels without evaporation problems or high thermal inertia unlike aquatic organisms. The use of calorimetric methods in the toxicity studies on insects enables, apart from others, the quick detection of the effect of toxicants on the nearly motionless developmental stage, the pupa, which otherwise would be difficult to evaluate. Bioassay methods mainly dependent on the visual evaluation of activity of an organism allow us to assess the action of toxicants only by counting the number of inactivated (dead) individuals but not the extent of poisoning on the surviving and weakened organisms. Calorimetric methods, however, help us in elucidating the extent of weakening of insects due to poisoning by sublethal doses of toxicants.

Our aim is to microcalorimetrically investigate the toxicity of propolis (bee glue) on the three developmental stages: larvae, pupae and adults of the yellow meal worm *Tenebrio molitor* L. (Coleoptera: Tenebrionidae).

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Propolis is a resinous natural product that honeybees collect from plants, mix with wax and use in the construction and modification of their hives. Chemically, propolis is a complex mixture of many compounds [10]. These components vary based on the geographical origin of the sample, since different plant exudates and secretions can serve as sources of propolis in different ecosystems. In a recent review on the chemistry and plant origin of propolis, it has been asserted [11] that propolis from different geographical origins may contain totally different chemical components. Even though the chemical makeup of propolis varies highly, its role in the beehive is universal. Bees apply propolis as a thin layer on the internal walls of their hives or other cavity they inhabit. It is used to block holes and cracks, to repair combs, to strengthen the thin borders of the comb, and to make the entrance of the hive weathertight or easier to defend. Bees also use propolis as an "embalming" substance to cover hive intruders, which are killed inside the hive but could not be transported out [10]. This action of embalming dead intruders contains putrefaction and the spread of disease in the beehive. Apart from the purely mechanical use of the glue-like and cementing properties of propolis, its use may also have a chemical basis, the volatile [12] and water soluble components of propolis being responsible for the lower incidence of bacteria and fungi within the apiary. In addition to its well-established antibacterial, antifungal and antiviral activities [10,13,14], the anaesthetic effect of propolis on rabbit cornea and frogs has already been reported [15,16]. The anaesthetic and acaricidal actions of propolis against the parasitic mite *Varroa destructor* Anderson and Trueman (formerly called *Varroa jacobsoni* Oud.) has already been established [17]. It has been proposed [18] that some flavonoid components of propolis could have insecticidal or at least insectistatic (inhibition of insect larval development) effects, which were not well investigated.

2. Experimental

2.1. Propolis extraction and preparation

Propolis samples used in our experiments were obtained from the research beehives in the Garden of the Institute of Zoology, Free University of Berlin, Germany. Frozen propolis samples were homogenized using a coffee mill (type MZ Moulinex, France) for further processing. The extraction was done in 70% ethanol according to a previously established method [19].

The dried 70% ethanol extract was dissolved and used in 55% ethanol in the bioassay. Even though 70% ethanol was used for extraction purpose, 55% ethanol was employed as a solvent in the bioassay in order to reduce the effect of strong ethanol solution on the experimental organisms. The little amount of precipitation observed while suspending the 70% extract in 55% ethanol was brought into solution by agitation.

2.2. Animals and treatment

Yellow meal worm (*T. molitor* L.) was reared in a plastic bowl on rolled oats at 27 °C and 70 ± 5% RH in constant darkness as described in the literature [7]. Larvae, pupae and adults weighing ≥175, 150 and 120 mg, respectively, were used in the experiments. Newly moulted larvae and pupae recognized by their pale colour and also highly irritable pupae were excluded from the experiments. The degree of irritability of the pupae was assessed by gently prodding them with a probe. Those pupae that showed wriggling movements up on jabbing were considered highly irritable and thus excluded.

The treatment with the given propolis concentration was done by dipping the experimental organism in the propolis solution for 60 s. Reference insects were dipped in 55% ethanol solution and also in distilled water for the same length of time. After the allocated treatment time, the organisms were put on a pad of paper towel (Kimwipes™ Lite 200, Kimberly-Clark™) and rolled gently to remove the excess fluid from the surface.

2.3. Petri dish bioassay

Since preliminary experimental results showed that propolis anaesthetizes the animals in the larval stage, 10 treated larvae per experiment were put on a clean Petri dish and their activity was observed in an interval of 1 h starting at zero observation time. After activity of the treated larvae was observed for 6 h, they were then incubated further for 15 h at 27 °C and

70 ± 5% RH on rolled oats, to avoid starvation, and final observation was done. Since the differentiation between anaesthetized and normal pupae was difficult, and complete anaesthesia was not observed in adults, this experiment was done only with the larva stage.

An individual was considered completely anaesthetized if it showed no leg movement and/or movement of any other body part when gently prodded with a probe. If it showed movement, whether it was partially paralysed or normal, it was counted as active. Each treatment was repeated three times and the mean values were used in the presentation of results.

2.4. Calorimetric measurements

The calorimeters used were: (i) a Biocalorimeter, BCP-600, Messgeräte Vertrieb, München, Germany with a sensitivity of 50 μV/mW and a vessel volume of 20 cm^3; (ii) a Calvet calorimeter, Setaram, Lyon, France, of vessel volume 100 cm^3 and sensitivity of 15 μV/mW.

In order to compare the metabolic rate before and after treatment and to assess the impact of a certain concentration of propolis, the heat production rate of an untreated organism was recorded for 3–4 h. Recording was then stopped, and the organism was removed from the calorimeter and treated with propolis as described above. The treated and blotted organism was put back in the calorimetric vessel and the heat production rate was recorded for 5–10 h. Recording of the heat production rate after treatment was run for longer period than before treatment since: (a) the moisture on surface of the organism, introduced due to treatment, interferes with the calorimetric signal because of evaporational heat loss and needs some time to diminish; (b) while recovering from narcosis the organism's physiological activity may change through time after treatment until it reaches its "after treatment steady state" activity status. Due to these reasons only part of the power–time (*p–t*) curve after attainment of a steady state heat production rate was considered in the interpretation of results. Each experiment was repeated five times and the mean ± S.D. values were used in the presentation of results. Control experiments for each experimental group were done by treating the organisms, for the corresponding time, with 55% ethanol and also with distilled water.

3. Results

Dipping the larvae in propolis for 60 s resulted in 100% narcosis immediately after treatment regardless of the concentration of propolis and even in case of the control experiment. The larvae treated with ethanol only (control) and 1% propolis recovered sooner, and only 3.3% of the larvae remained narcotised for the first 6 h after treatment. The percentage of larvae that recovered from narcosis after treatment decreased considerably with increasing concentrations of propolis. Narcosis due to treatment with stronger concentrations of propolis lasted longer. Further incubation showed that some of the larvae that recovered from narcosis in the first 6 h died in the next 15 h, elevating the percentage of inactivated larvae (Fig. 1). The larvae that did not recover within 21 h of treatment were dead, witnessed by the oxidation and browning of their tissue. Even the larvae that recovered from narcosis were very weak and unable to perform their normal locomotion.

The *p–t* curves obtained from untreated larvae and adults showed irregularity and larger differences between the maximum and minimum points of the *p–t* curves (Fig. 2). The *p–t* curves of the untreated pupal stages, however, were more or less regularly structured and the differences between the maximum and minimum points of the *p–t* curves were smaller (Fig. 2). Treatment of the larvae and pupae with 5% propolis in 55% ethanol resulted in a drastic drop in the heat production rate, by 75 and 83%, respectively, and the *p–t* curve became very smooth (Fig. 2). The treatment of the adults with the same concentration of propolis, however, did not have a considerable impact on the minimum points of the *p–t* curve (heat production rate in the absence of locomotion), which were reduced by about 10%. The heat production rate due to muscular contraction and locomotion (peaks of the *p–t* curves) of the adults after treatment with 5% propolis was reduced by about 26% and the interval between bouts became longer (Fig. 2).

The dose-response curves for the treatment of larvae and pupae with different concentrations of propolis showed similar patterns (Figs. 3 and 4), with the reduction of the mean heat production rate by more than 90% due to treatment with propolis concentration of ≥7.5%. Treatment of the adult stage, however, even with 10% propolis resulted in a reduction of the mean

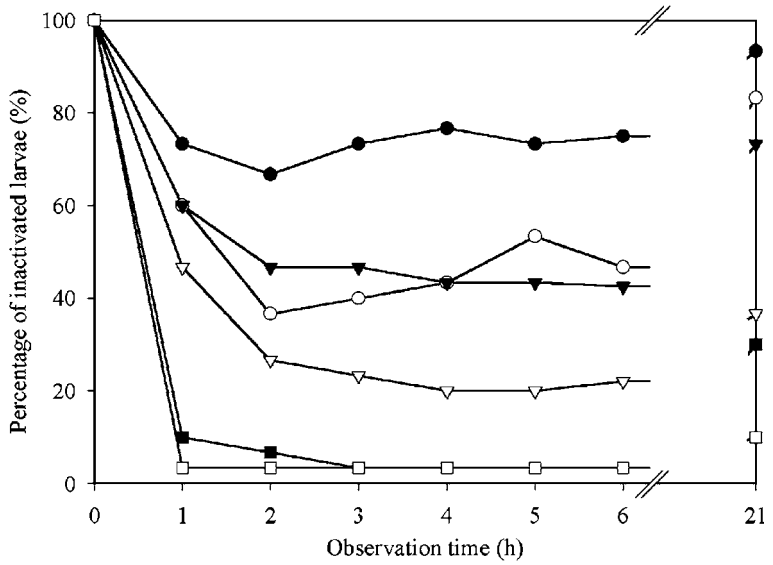

Fig. 1. Percentage of inactivated larvae of *T. molitor* L. after treatment, by dipping for 60 s, with different concentrations of propolis in 55% ethanol. Ten larvae per experiment, $n = 3$ for each concentration. Propolis concentrations used were: ●—10%, ○—7.5%, ▼—5%, ▽—2%, ■—1%, □—Control.

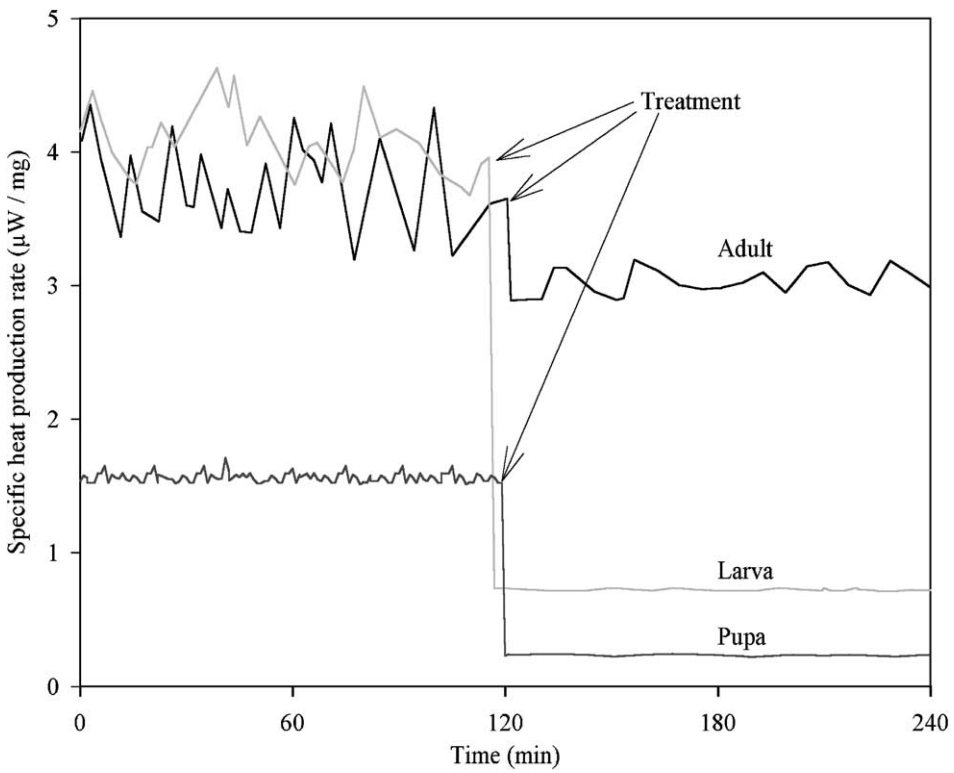

Fig. 2. Effect of treatment of *T. molitor* L. larvae, pupae, and adults with 5% propolis in 55% ethanol on the structuring of the power–time curve.

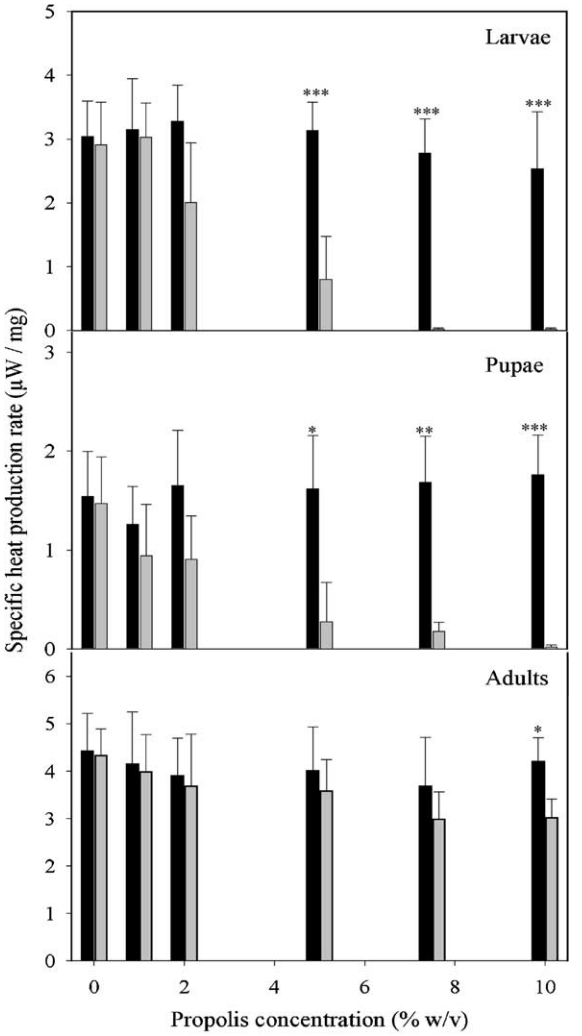

Fig. 3. Effect of treatment of *T. molitor* L. larvae, pupae, and adults with different concentrations of propolis in 55% ethanol on the specific heat production rate (μW/mg). $n = 5$ for each concentration. Significance levels of *—$P < 0.05$, **—$P < 0.01$, ***—$P < 0.001$ (paired sample *t*-test). ■—Before treatment, □—after treatment.

specific heat production rate by only 28%, with lower concentrations of propolis having no significant impact (Fig. 3). The treatment with alcohol (control experiment) did not display a significant reduction ($P > 0.05$) in the heat production rate, confirming that the alcohol does not have a considerable effect on the experimental organisms.

4. Discussion

The topical application of propolis on the larval stage of *T. molitor* L. showed both narcosis and death, the extent of narcosis and eventual death being dependent on the concentration of propolis. Even though the larvae were observed getting out of narcosis, moving their appendages when slightly prodded, further incubation, for a total of 21 h, displayed that the proportion of dead larvae was increasing. This may be due to the fact that the different components of propolis played different roles: some of the components being responsible for the short lasting anaesthesia, while the other components being responsible for the long-term poisoning and death. The anaesthetic action of propolis observed in *T. molitor* L. seems to agree with previous results obtained with different groups of animals by topical application: anaesthetic and lethal action on *Varroa destructor* Anderson and Trueman [17], anaesthesia of the rabbit cornea [15,16], surface anaesthesia of frogs [16] and anaesthesia of the human cornea [20]. The components of propolis that are responsible for anaesthesia in different groups of animals include essential oils [16], flavonoids [18] and the water extracts [20].

Narcotic experiments were difficult to do with the pupal stages since they lack locomotion making the evaluation difficult. Complete narcosis was not observed in the adults, at least with the range of propolis concentration used, indicating that the strong cuticular layer impedes penetration of the propolis solution. Even though it was difficult to evaluate the effect of propolis on pupae and adults by the narcosis experiments, it was accomplished by the calorimetric method, making the latter superior in the investigation of effects of sublethal toxicants on organisms [7,17,20].

Calorimetric recordings after treatment with 5% propolis displayed that the latter has a strong influence on the structuring of the *p–t* curve and reduces the heat production rate drastically, especially in case of larvae and pupae. Even though the standard heat production rate of adults after treatment with 5% propolis dropped by only 10%, the heat production rate due to contraction of muscles and locomotion, the peaks on the *p–t* curve, dropped by 26% indicating that propolis anaesthetizes the organisms rendering them motionless or performing feeble locomotion. The

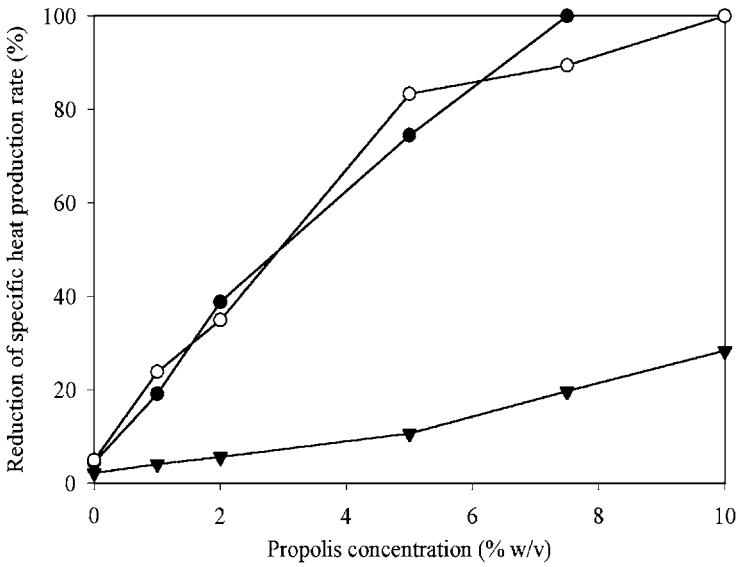

Fig. 4. Reduction of the specific heat production rate of the three developmental stages of *T. molitor* L. after the treatment with different concentrations of propolis in 55% ethanol. $n = 5$ for each concentration, ●—larva, ○—pupa, ▼—adult.

weak poisoning action of propolis on adults, as compared to larvae and pupae, may be due to the lower permeability of the thick cuticular layer of the adults to propolis, rendering it as a surface anaesthetic, as already displayed in frogs [16]. In case of larvae and pupae, however, propolis has a stronger poisoning effect as their cuticle is considerably thinner.

The dose-response curves of treatments with different concentrations of propolis displayed the same pattern in larvae and pupae, indicating their similar sensitivity to toxicants since both have a thin cuticle.

The high sensitivity of the larval and pupal stages of *T. molitor* L. towards the alcohol extracts of propolis demonstrates that propolis can be used as an insecticide, mainly applied during these two developmental stages. The use of propolis as an insecticide may help us to minimize the ever-increasing problem of insecticide and pesticide resistance and the problem of environmental pollution due to the application of synthetic insecticides. The development of resistance against complex natural products, such as propolis, having different components with various modes of action is unlikely or very slow [21]. In addition, since propolis is a natural product, it may cause less residue problems unlike synthetic pesticides.

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