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Effect of the bee glue (propolis) on the calorimetrically measured metabolic rate and metamorphosis of the greater wax moth *Galleria mellonella*

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Abstract

Among the moth pests of the honeybee, the greater wax moth *Galleria mellonella* (Lepidoptera: Pyralidae) causes the greatest damage, unless controlled at an early stage, because it feeds on wax, pollen, and cocoon of the bee larvae. This leads to the destruction of honeycomb and subsequent deterioration of weakened colonies. For controlling the pest, natural products are second to none, not least because the use of synthetic substances carries with it the problem of residues, which remain in the beehive to affect the bee products. This paper reports the results of calorimetric investigations on the effects of the bee natural insecticidal glue, propolis, on pupal metamorphosis and the metabolic rate of different larval instars.

Experiments were performed by batch calorimetry to record the heat flow rate of individual larvae/pupae before and after the treatment, which consisted of dipping L5, L6, and L7 instars in a graded series of different concentrations of ethanol-dissolved propolis for 30 s before blotting them. The heat production rates were then recorded for 6–7 h (short period experiment) or during the entire pupal metamorphosis (long period experiment).

The fifth larval instar (L5) showed higher sensitivity to propolis treatment than L6 and L7 whereby total mortality was obtained by 4% propolis for L5 and 8–10% for the latter. The higher sensitivity of L5 can be accounted for by the very high mass-specific metabolic rate and the thinner and more fragile cuticle, typical of early larval stages, allowing the free transit of nonpolar toxic substances from the surroundings after being easily disrupted by components of propolis.

The treatment of the late L7 stage with nonlethal doses of propolis shortened the duration of pupal metamorphosis significantly. An untreated larva required 6.8 ± 0.8 days (mean $\pm$ S.E., $n = 5$) between larval–pupal and pupal–adult ecdysis, whereas this time was shortened to 5.4 ± 0.9 and 4.8 ± 0.5 days after treatment with 1 and 2% propolis, respectively. Though all treated larvae went through larval–pupal ecdysis, 40 and 100% of those treated with 2 and 4% propolis, respectively, displayed abortion of pupal metamorphosis and died.

These results indicate that propolis is toxic at higher concentrations and an insect growth regulator at lower ones. The use of propolis in the control of *G. mellonella* and its subsequent occurrence in honeybee products such as honey and wax may not cause the problem of a toxic residue, as it is the natural component in the beehive.

Keywords: *Galleria mellonella*; Propolis; Insecticide; Wax moth; Calorimetry

1. Introduction

Among the wax moth pests of the honeybee the greater wax moth *Galleria mellonella* (Lepidoptera: Pyralidae) causes the greatest damage, leading to material and financial losses. The larval stage of *G. mellonella* (with its seven instars), the only feeding stage with the longest life span of all developmental stages, builds its silk-lined feeding tunnel in the honeycomb and feeds on wax, pollen, feces and cocoon of the bee larvae. This voracious nature of the larva leads to the destruction of the honeycomb and the subsequent death of weak colonies. Adults do not feed because they have atrophied mouth parts.

The greater wax moth can be controlled by biological, physical and chemical methods, but most of these methods are either inefficient or expensive to the small-scale

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beekeeper. In addition to that, the chemical methods cause problems of residues remaining in honeybee products [1].

An alternative, and most likely the best solution, to solve the residue problems associated with most chemical treatments and the financial costs incurred by most physical and biological control methods could be the use of natural products that are at hand to the beekeeper and free of the aforementioned problems. One such honeybee product is propolis.

Propolis or bee glue is a brown resinous substance collected by honeybees from various plants and mixed with wax and salivary gland secretions of the bees. The different components of propolis are produced by plants in order to avoid infection of injured tree parts and help ward off or kill insects or mite pests [2]. Propolis is mainly used by bees to protect the hive against infection and also as a multipurpose cement and varnish. Though propolis is found inside the beehive, it does not play a significant role against the pathogens and pests of honeybees in situ. However, in vitro experiments demonstrated that propolis is varroacidal (kills Varroa mites) [3,4], bactericidal and fungicidal—kills several microbes including the bacterial pathogen that causes foulbrood [5,6].

The potential residue free use of propolis, compared to the commercially available expensive, hazardous and residue-associated insecticidal agents employed in the combat against *G. mellonella* provide an incentive to investigate its insecticidal action against this wax moth.

As to the method used, all investigations were done using calorimetry. Calorimetry is a useful technique in the continuous monitoring of different developmental processes throughout the whole life cycle of individual insects, as it reveals metabolic events that cannot be detected by some standard methods. Several researchers have employed calorimetry in the investigation of insect growth and development; among others for the well-studied *G. mellonella* by [7–12]. In addition to that, the insect growth regulator (IGR) and toxic effects of plant secondary metabolites on insects have been investigated calorimetrically [13]. Standard bioassay methods, for example, Petridish bioassay, demonstrate the results of extreme cases of biological activity, such as lethality of a certain concentration, or its impotence demonstrated by the survival of the organism after treatment. Biological activities of sublethal concentrations and their effects on further development of the organism could, however, be online monitored by the use of the calorimetric method that detects heat production rate, which is in turn directly determined by the metabolic rate. This latter method was found to be highly sensitive in the investigations of effects of plant secondary metabolites on insect metamorphosis [13] and on the *Varroa* weakening action of propolis [3]. In addition to its high sensitivity, the calorimetric method enables one to judge the mode of action of an insecticidal/insectstatic agent.

The aim of our investigation was to calorimetrically demonstrate the insecticidal and/or insectstatic (abort insect larval/pupal development) action of propolis.

2. Experimental

2.1. Animal and culture conditions

The greater wax moth *G. mellonella* was cultured in a plastic bowl (25 cm × 25 cm × 10 cm) at ambient temperature of 30 °C, relative humidity ca. 70% and 24 h darkness. The culture medium (larval food) consisted of 22% maize flour, 11% wheat flour, 11% bruised wheat, 11% milk powder, 5.5% yeast, 17.5% beeswax, 11% honey, 11% glycerine. All the larval stages and eggs were kept together separated from the pupal and adult stages.

As the early larval stages are too small to handle and too delicate to be used for the purpose of the present investigations, only the fifth, sixth, and seventh larval instars L5, L6 and L7, respectively, were chosen. Identification of each larval instar was done by the width of the head capsule and its weight as parameters given in the literature [14]. Freshly moulted larvae, identified by their creamy white color, were not included in the experiments, since they have weaker cuticles and may introduce bias in the results.

2.2. Calorimetric experiments

The calorimetric experiments were performed using three isoperibolic heat-conduction differential batch calorimeters with different vessel volumes. All investigations with L5 were in an instrument (Biocalorimeter B.C.P-600, Thermanalyse, München, Germany) of vessel volume 12 ml, and a sensitivity of 44.7 μV/mW. For corresponding experiments with L6 and L7 larvae, two Calvet calorimeters (SETARAM, Lyon) with vessel volumes of 15 and 100 ml, respectively, were used. Each of these calorimeters has two measuring and two reference vessels. The sensitivities of the instruments amounted to 62.6 and 44.2 μV/mW for the two vessels with volume of 15 ml and 51.5 and 53.7 μV/mW for the two vessels with volume of 100 ml.

To avoid starvation and behavioral change, the larvae were provided with sufficient food for the entire experimental period. Two types of calorimetric investigations were performed: short and long-time experiments.

The presence of the larval food, in both the measuring and reference chambers, does not interfere with baseline stability, i.e. even though there could be heat generation, it is cancelled by the differential arrangement of the measuring and reference vessels. Since the calorimetric vessels, made of Pyrex glass, are open, there is no oxygen limitation.

Short period experiments. The aim of these experiments was to investigate the effect of different sublethal concentrations of propolis on the heat production rate of the three larval stages mentioned (L5, L6 and L7), and compare the change in the sensitivity to propolis, if any, with changing larval instar. Both the measuring and reference vessels were supplied with equal amount of food to avoid asymmetry of nonexperimental factors in the two vessels. After establishment of the baseline, a pre-weighed larva was placed into the

measuring vessel and the heat production rate was recorded for ca. 4 h. Then the larva was removed from the vessel and treated with propolis, as described below. The treated larva was put back into the calorimeter and the heat production rate recorded again for 6–7 h. Each experiment was done six times and results are presented as mean ± S.E.

Long period experiments. These experiments were done only with L7. The aim was the evaluation of the effect of sublethal concentrations of propolis on metamorphosis and development of the pupal stage. This could answer the query whether sublethal concentrations without remarkable effects on the larva could cause abortion of pupal development or either shorten or prolong the pupal development time. The heat production rate of the untreated, pre-weighed larva was recorded for 1 day in order to observe its activity before treatment. The larva was removed from the calorimeter, weighed again, treated with the desired propolis concentration and put back into the calorimeter. The heat production rate was recorded further until adult emergence, with weight measurements every 24 h. The mean weight between two consecutive weighings was used in the calculation of the specific heat production rate in this period (24 h). In cases where there was no adult emergence, recording was continued for a total of 25–30 days and finally the calorimetric vessel was opened, the pupa removed and inspected for life under a binocular microscope by pricking with a blunt needle. The maintenance of a constant weight during pupal development was also used as a preliminary clue of the death of the organism. Each treatment, including the controls, was done five times and the values are presented as mean ± S.E.

2.3. Propolis preparation and larval treatment

Propolis samples obtained from the research beehives of the Institute of Zoology, Free University of Berlin, were extracted in 70% ethanol and prepared according to a previously established method [4]. From the extracted and dried propolis sample, a 10% (w/v) propolis stock solution was prepared in 55% (v/v) ethanol. The desired concentrations for treatment (0.25, 0.5, 1.0, 2.0, 4.0, 8.0 and 10.0%, w/v) were obtained by diluting the stock solution with 55% ethanol.

Treatment was done by dipping the larvae in 5 ml propolis solution in a 30 ml vial for 30 s. After the allocated treatment time, the larvae were removed with a pair of tweezers with extreme caution not to damage them, and placed for 1 min on a pad of absorbent paper towels to blot fluid, which could disturb the calorimetric signal and prolong the experimental time undesirably. Double control experiments were done by dipping the larvae in 55% ethanol and distilled water. After being properly blotted, the larvae were put back into the calorimeter and recording the heat production rate started after a thermal equilibration time of 30–45 min, which is always needed after replacing the calorimetric vessel.

3. Results

Unless otherwise clearly stated, all values in this work are given as mean ± S.E.

The wet weight of the larval instars increased drastically, from a mean value of 23.0±2.5 mg at L5, to 65.7±5.8 mg at L6, achieving its maximum mean value of 236.8±48.1 mg at L7 (the seventh larval instar). These values are means of the larval instars used in the present investigation. Otherwise, the weight change during the entire larval developmental stage ranges from <1 mg for L1, to nearly 400 mg at L7 of some individuals. A nearly uniform drop of weight during pupal metamorphosis then followed this drastic increase, as it will be seen in the next sections.

The mean total heat production rates of untreated larvae increased with larval age from L5 (1.7 ± 0.2 mW) to L7 (6.5 ± 0.4 mW) and dropped drastically at the pupal stage (1.9 ± 0.3 mW). The specific heat production rates, however, followed a reverse pattern, except at the pupal stage, dropping considerably from L5 (78.9 ± 8.9 mW g^{-1}) to the pupal stage (8.2 ± 3.1 mW g^{-1}) (Fig. 1).

The fifth larval instar was highly sensitive to propolis treatment compared to the sixth and seventh instars. Whereas the two latter instars did not display sensitivity even to 0.5% propolis, the heat production rate of the fifth larval instar was reduced by 48% due to treatment with 0.25% propolis (Fig. 2). Treatment with 4% propolis resulted in 100% mortality of L5 and reduced the heat production rate of L6 and L7 to 25–30% of the initial value (Fig. 2). Although the heat production rate of L5 dropped by 7.4%, this change was not statistically significant (paired sample *t*-test, $P = 0.09$); and the other two larval instars did not show any observable sensitivity to the control treatment (Fig. 2). The sixth and seventh larval instars were sensitive to treatments with propolis concentrations of 1%, with 10% propolis resulting in 100% mortality of L7 and a consequential drop of the specific power–time (*p*–*t*) curve to the baseline, represented by the nil bar for the mean heat production rate in Fig. 2. The same concentration reduced the mean heat production rate of L6 by 94% (Fig. 2). In addition to the change in the mean heat production rate, the change in the mean mass-specific heat production rate is also displayed here for each larval stage, and both show similar patterns (cf. Figs. 2 and 3). The dose-response curves of residual mass-specific heat production rate vs. propolis concentration are the same for L6 and L7, but L5 displays a different pattern (Fig. 4). Percentage residual heat production rate is used here to refer to the heat production rate after treatment divided by that before treatment and multiplied by 100.

The typical long period *p*–*t* curve of *G. mellonella* development in the last larval and the pupal stage showed a drastic drop in the heat production rate from the late L7 to the pupal stage through the prepupal phase (late L7 enclosed in the silk cocoon) (Fig. 5a). This pattern was observed for the untreated, and also the blank solution treated (control) organisms. The transition from prepupa to the pupal phase

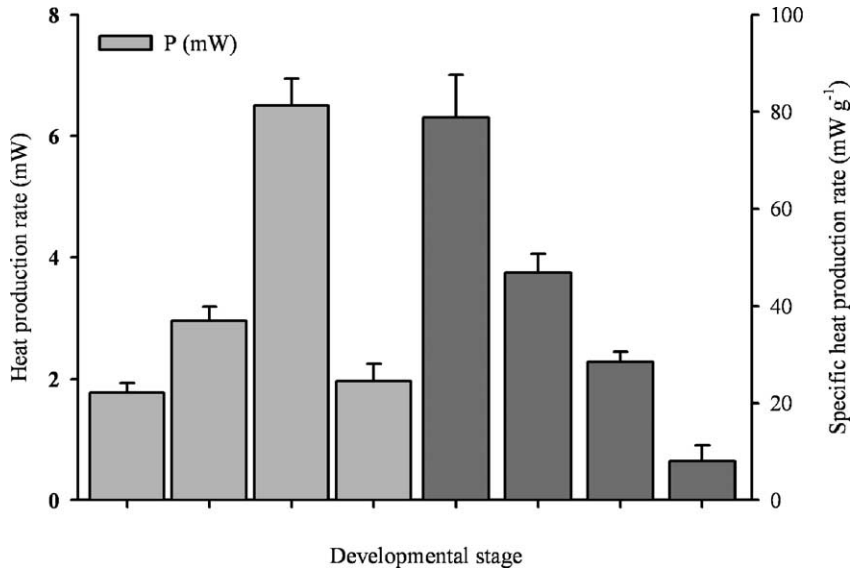


Fig. 1. Heat production rate (P, mW) per individual larva and mass-specific heat production rate (p, mW g^{-1}) of L5, L6, L7 and pupa of the greater wax moth *G. mellonella*. L5, L6 and L7 represent the fifth, sixth and seventh instar larvae, respectively. Mean ± S.E., $n = 54$ for L5 and pupa, and $n = 36$ for L6 and L7 each.

was accompanied by a sharp peak followed by a trough at ecdysis. The pupal heat production rate then dropped from a mean of 2.2 ± 0.9 to 0.9 ± 0.3 mW in 24 h and remained at this level for the next 3 days. The heat production rate started to ascend between the third and fourth days after pupation and achieved a maximum value of 3.6 ± 0.8 mW on the sixth day. The pupa–adult molting occurred 6.6 ± 0.7 days after the larva–pupa molting. This last molting was

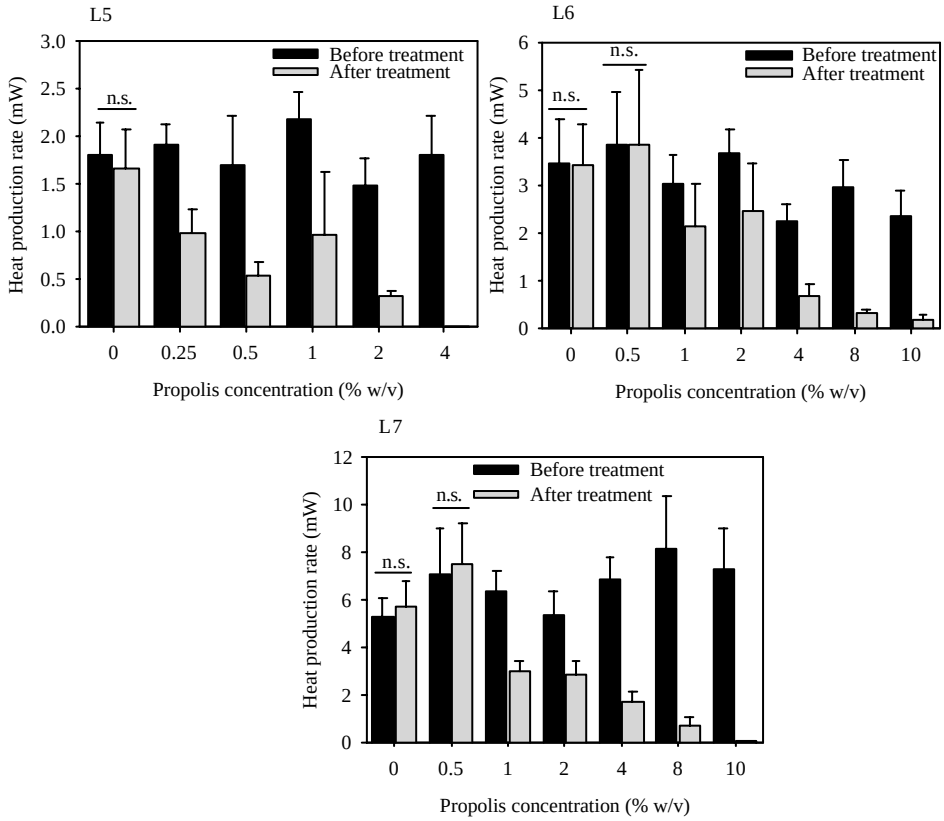


Fig. 2. Effect of treatment on the heat production rate per animal of the different instar larvae: (a) L5, (b) L6 and (c) L7 of the greater wax moth *G. mellonella* with different concentrations of propolis, in 55% ethanol. Mean ± S.E., $n = 9$ (for L5) and $n = 6$ for L6 and L7. n.s.: no significant difference (t-test, $P > 0.05$).

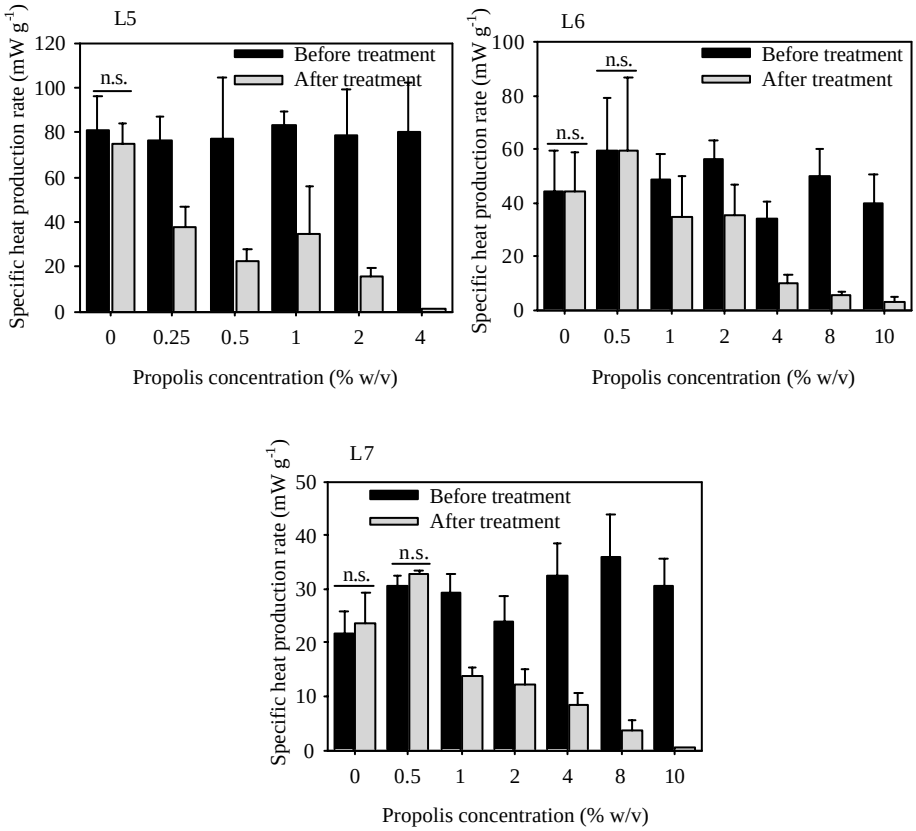


Fig. 3. Effect of treatment on the mass-specific heat production rate of the different instar larvae: (a) L5, (b) L6 and (c) L7 of the greater wax moth *G. mellonella* with different concentrations of propolis, in 55% ethanol. Mean ± S.E., $n = 9$ (for L5) and $n = 6$ for L6 and L7. n.s.: no significant difference (*t*-test, $P > 0.05$).

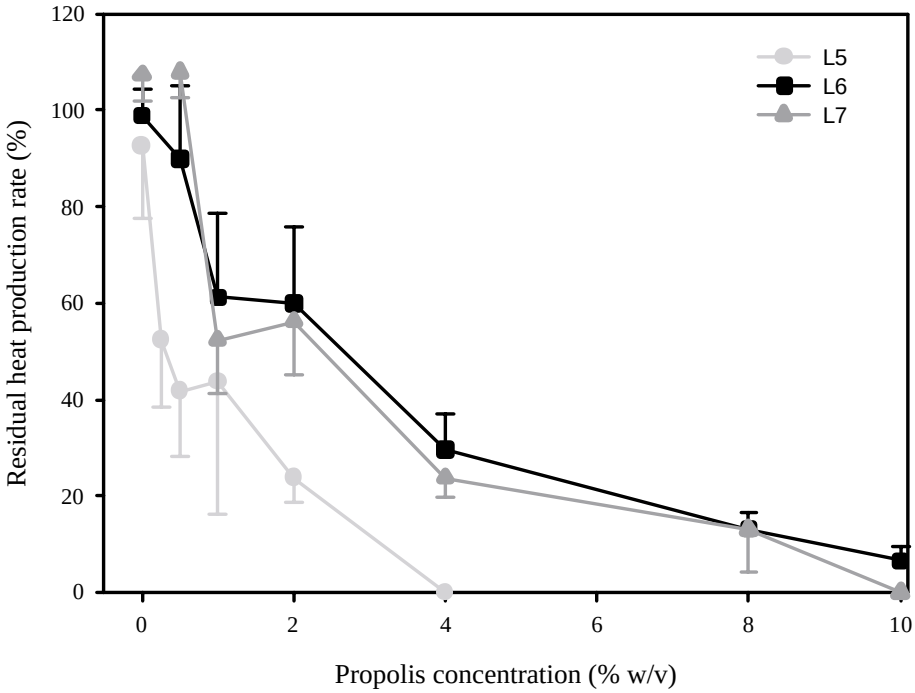


Fig. 4. Percentage residual (specific) heat production rates of the fifth (L5), sixth (L6) and seventh (L7) larval instars of the greater wax moth *G. mellonella* after treatment with various concentrations of propolis in 55% ethanol. Mean ± S.E., $n = 9$ (L5) and $n = 6$ (L6 and L7).

accomplished after a strong muscular contraction activity displayed by a sharp peak of 8.2 ± 0.45 mW followed by a trough of 0.2 ± 0.1 mW (Fig. 5a).

The five pupae treated with 1% propolis during the late L7 stage successfully completed their development to adult emergence whereas only 60 and 0% of those treated with 2 and 4% propolis, respectively, were successful. The peaks and troughs during ecdyses of treated pupae were not as strong as in the case of the controls. In addition to that, the emerged adults did not show the typical *p–t* curve displayed by the controls; they rather displayed weak locomotory (flying) activities (cf. Fig. 5a–c). The pupal metamorphotic phase lasted 5.38 ± 0.9 days after treatment with 1% and 4.8 ± 0.5 days with 2% propolis.

Though the larvae treated with 4% propolis performed the larva–pupa ecdyses, the pupal development was aborted in

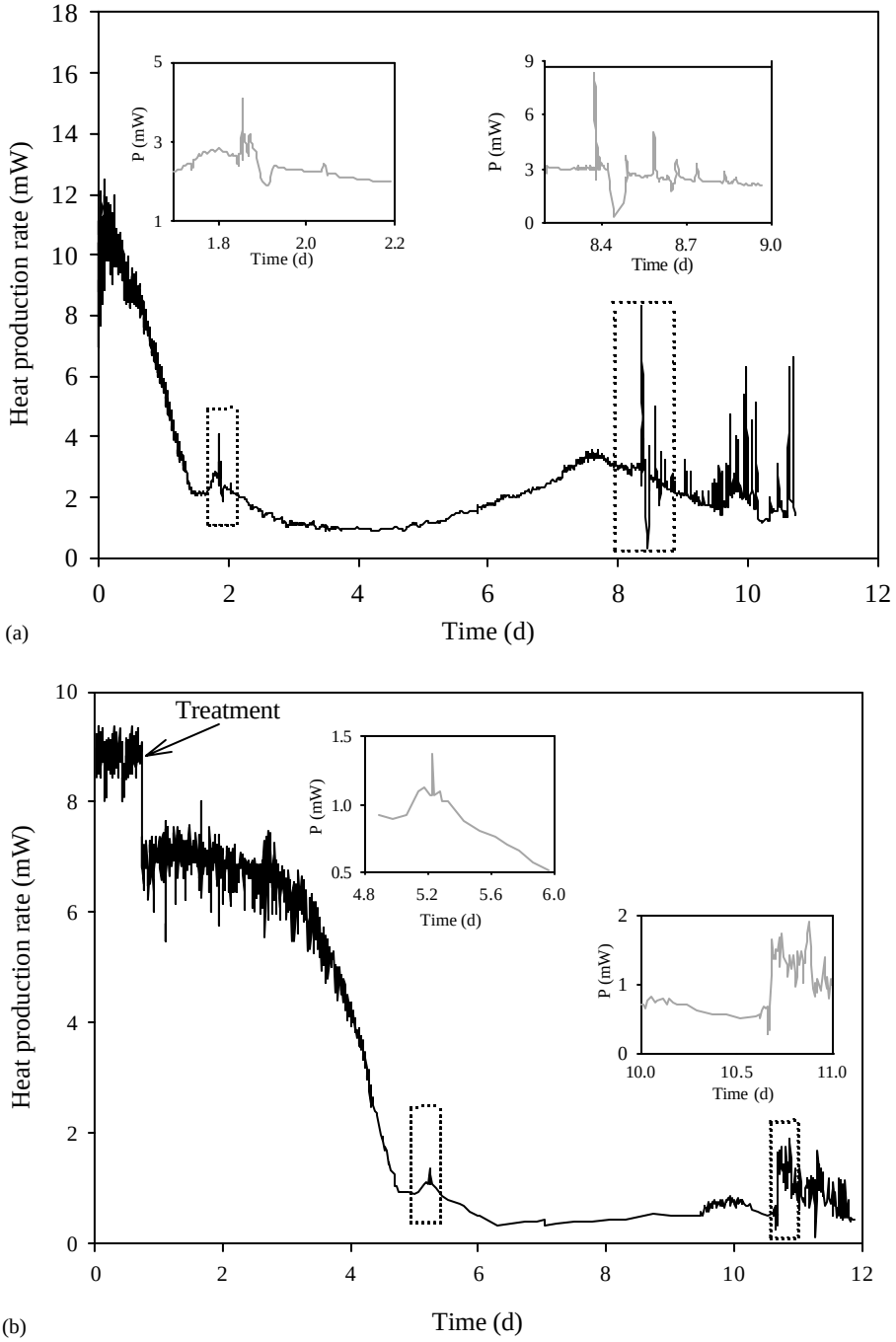
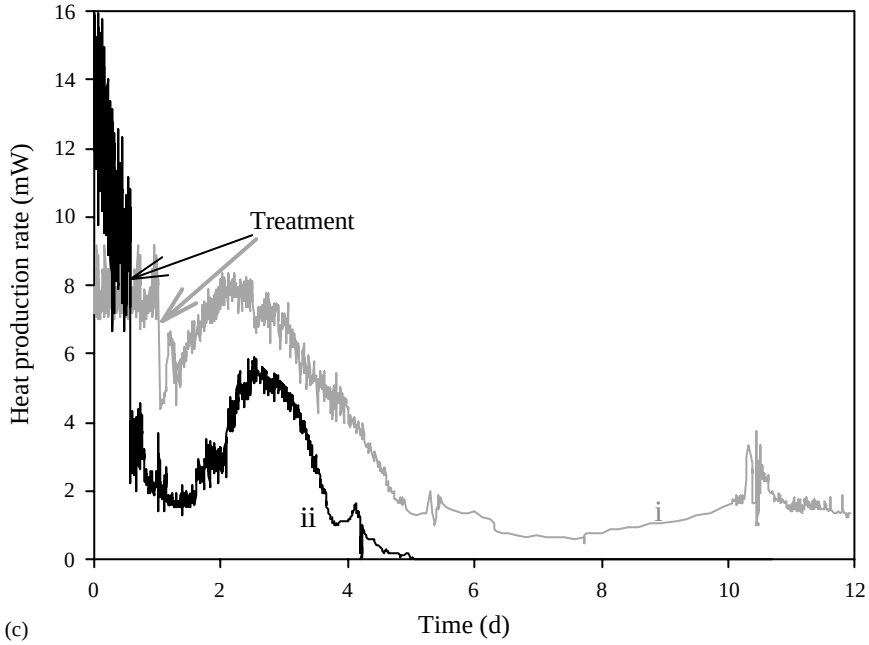
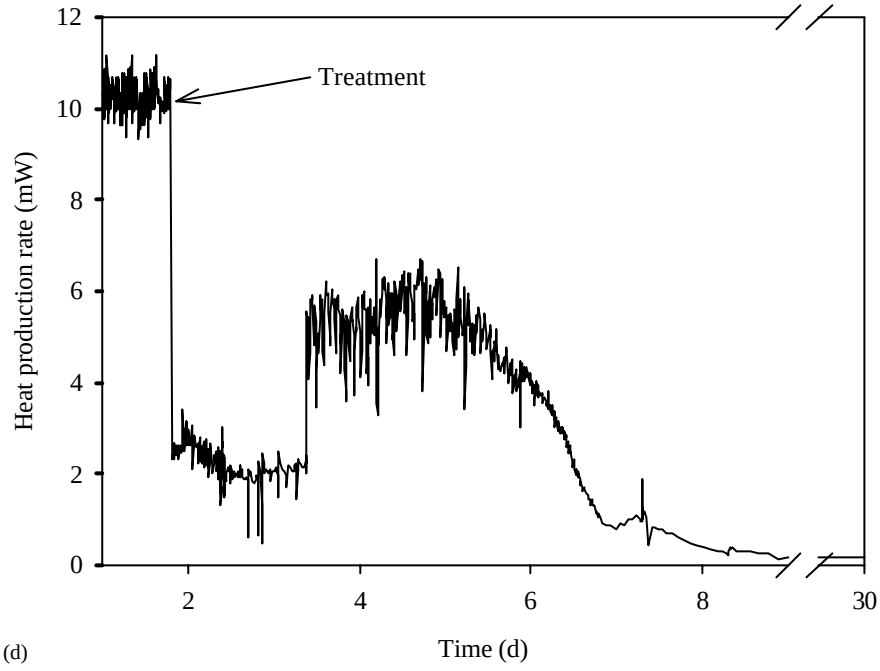


Fig. 5. Typical power–time (*p–t*) curves of the development of *G. mellonella* from the late seventh larval instar to adult emergence: (a) 307 mg larva without treatment; (b) 173 mg larva treated with 1% propolis; (c) 209 mg (i) and 308 mg larvae (ii) treated with 2% propolis; (d) 189 mg larva treated with 4% propolis. Note the differing vertical scales. Treatment period was 30 s. The insets are enlarged portions of the larval–pupal and pupal–adult ecdysis, marked by rectangles on the curve underneath the corresponding inset.



(c)



(d)

Fig. 5. (*Continued*).

all the five larvae investigated. These results indicate that, though the larvae survived the treatment and had a percentage residual heat production rate of ca. 25% (Fig. 4), they were too weak and too unhealthy to go through pupation.

Apart from the differences in the height of peaks and the depth of troughs associated with molting, the treatment with different concentrations of propolis introduced a significant difference in the length of the pupal metamorphotic phase. Metamorphosis of a pupa not treated during the larval stage lasted 6.6 ± 0.7 days and that of the control treatment, treated with a blank solution (55% ethanol), lasted 6.8 ± 0.8 days, not significantly different from each other (*t*-test, $P = 0.25$). The treatment with 1 and 2% propolis reduced the length of metamorphosis to 5.4 ± 0.9, and 4.8 ± 0.5 days, respectively. A one way ANOVA followed by Tukey's test ($n = 5, \alpha = 0.05$) demonstrated that the length of metamorphosis after treatment with 1 and 2% propolis are not significantly different from each other ($P = 0.09$) but do differ significantly from the two control treatments ($P < 0.05$).

The specific heat production rate during pupal development showed a typical U-shaped curve for the controls. The

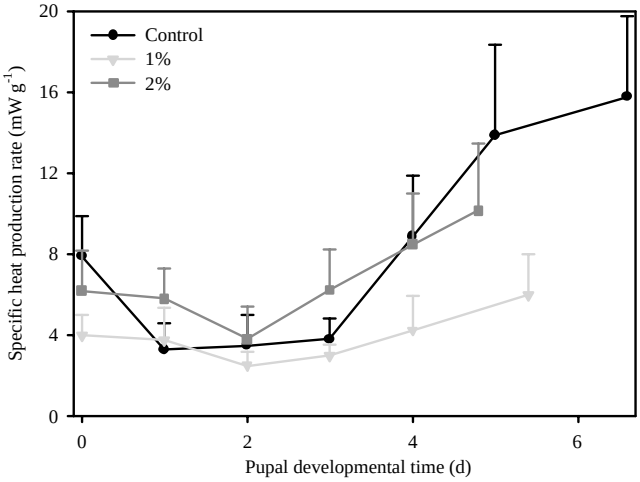


Fig. 6. Rate of mass-specific heat production (mW g^{-1}) of the greater wax moth *G. mellonella* during pupal development, after treatment of the seventh instar larva with different concentrations of propolis. The pupal developmental time is counted starting from the larval–pupal molting day as zero. Mean ± S.E., $n = 5$.

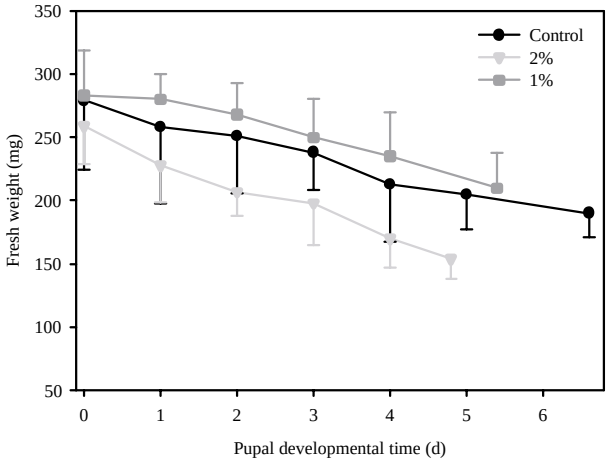


Fig. 7. Change of weight of the metamorphosing pupa of the greater wax moth *G. mellonella* after treatment with different concentrations of propolis at the seventh larval instar. Only pupae that successfully completed pupal development were considered. The day of larval–pupal molting was regarded as day zero. Mean ± S.E., $n = 5$.

curves for the treated pupae were, however, flatter with a smaller heat production rate in the late pupal and adult stages (Fig. 6).

The change of weight during the pupal development, displayed similar patterns regardless of the treatment (Fig. 7). All pupae investigated showed a uniform loss of weight with developmental time until adult emergence.

4. Discussion

The wet weight of larvae increased exponentially from the fifth to the seventh larval instar and dropped uniformly during pupal metamorphosis. This is because the larval stage is a "feeding machine", continuously consuming available food in order to accumulate enough reserve food for the entire phase of pupal metamorphosis and for the flying and reproductive activity of adults. As the pupal phase does not feed and hence completely depends on the reserve food accumulated during the feeding larval stage, its weight decreases at a constant rate during metamorphosis. The uniform drop in the weight of the pupa during metamorphosis indicates that the rate of consumption of reserve food during this phase of *Galleria* development is uniform. The tissue composition (proportion of fat, proteins and carbohydrates) remains almost constant at the various larval and pupal stages, changing only in the adults with increasing fat proportion [15].

The heat production rate increased from 1.77 ± 0.17 for L5 to 6.51 ± 0.44 for L7 mainly due to the drastic increase in the wet mass. However, the mass-specific heat production rate decreased from 78.8 ± 8.8 for L5 to 28.6 ± 2.0 for L7 and 8.2 ± 3.1 for the pupal stage. This decrease in the mass-specific heat production rate is mainly due to the fact that the bulk of the increased weight of L6, L7 and the pupal stages is reserve food and not metabolizing tissue. In the fifth larval stage, the main component of the larval weight is metabolizing tissue, leading to a very high mass-specific heat production rate [8,15].

The higher sensitivity of the fifth larval instar to propolis treatment is due to at least two factors: the thin and relatively permeable cuticular layer and the very high mass-specific heat production rate, both leading to a greater penetration of propolis.

Since the larval cuticle, or exoskeleton, stretches only to a limited extent it must be shed periodically to accommodate the rapidly growing body size of the larva, which could double daily during the first 10 days under ideal conditions [16]. Though the basic outer layers of the new cuticle are formed before shedding the old one, additional layers of endocuticle are added and sclerotization of outer layers increases with developmental days, throughout the duration of the instar [17]. This indicates that the strength and thickness of the cuticle increases with age of the larval instar, the more the number of days in each instar the stronger is the cuticle. The life spans of L5, L6 and L7 under ideal conditions are 2.2, 3.0, and 7.5 days, respectively [14]. Thus, L5 has the thinnest cuticle with high permeability and L7 has the thickest cuticular layer, which impedes penetration of the lipophilic components of propolis. It is therefore highly plausible to state that the thickness of the larval exoskeleton could play a role on the insecticidal action of propolis.

The higher mass-specific heat production rate of the L5 plays a role in the faster penetration of propolis across the cuticle and hence accumulation of higher propolis concentrations. This is mainly due to an increased transport of hydrocarbons and lipids through lipid pore canals across the cuticular layer [18]. The same mechanism may allow for the penetration of nonpolar pesticides, since there is a correlation between active biosynthesis of hydrocarbons and transport to the surface and penetration by pesticides, especially

the nonpolar ones [19]. As the majority of bioactive components of propolis are nonpolar, the analogy of penetration of nonpolar pesticides and propolis across the cuticular layer is reasonable.

When molting, the cuticle begins to separate from the epidermis, the larva reduces feeding activity and becomes quiescent. Each active stage in the larval life is thus followed by a sluggish premolting period [20]. This quiescent stage is accompanied by the declining heat production rate and a "U-shaped" power–time (p–t) curve shortly before larval–pupal ecdysis. In this quiescent phase, part of the old cuticle is degraded, resorbed and recycled by the epidermal cells for the formation of the new cuticular layer. Final break up of the old exoskeleton is achieved by peristaltic contraction of abdominal muscles, raising blood pressure in the thorax and splitting the former at the weakest point, usually along the mid-dorsal line [17]. This contraction of abdominal muscles is accompanied by a sharp peak on the p–t curve, and the break of the old cuticle and subsequent release of exuvial fluid is shown by the trough of evaporational heat loss. The height and area of the sharp peak and the depth of the trough indicate the amount of energy spent on contraction of the muscles and evaporation of exuvial fluid, respectively.

The treatment with propolis disturbs the above described typical molting activity features of *G. mellonella*. After treatment with 1% propolis, all larvae were able to go through the metamorphotic phase and emerge as adult, but the peaks and troughs were smaller than those of the controls. The adult emerged after the unusual molting behavior displayed a very weak flying activity demonstrated by the form of the p–t curve (cf. Fig. 5a–c).

With increase in the concentration of propolis, the length of the pupal phase was shortened significantly from 6.8 ± 0.8 days (ethanol control) to 4.8 ± 0.5 days (2% propolis). This suggests that propolis accelerates the development of the larval/pupal stage of *G. mellonella*. The unusually higher rate of metamorphosis may lead to malformed and immature individuals.

The biological activity of propolis displayed on *G. mellonella* is comparable to that of IGR and toxicants calorimetrically investigated by several researchers. Among others, Kuusik et al. [13,21,22] elucidated that IGR and toxic compounds/mixtures interfere with the form of the p–t curve of insect development, even leading to abortion of metamorphosis. It was stated [23] that IGR could act to inhibit, retard or even accelerate insect developmental processes. The biological activity of propolis on *G. mellonella* obtained in the present investigation fits with those that accelerate insect development. It was also demonstrated by several researchers [24–27] that the application of IGR at the larval stage resulted in the disruption of pupal development and early adult emergence; and in addition, normal ecdysis was not achieved. Also, treated larvae may give rise to morphologically deformed adults that are unable to fly properly.

The use of moderate concentrations of propolis, such as 4%, in the control of *G. mellonella* is reasonable since it is toxic and kills the early larval stages immediately, facilitates larval–pupal ecdysis, and aborts pupal development of the late larval stages. The practical significance of such concentrations of propolis is that they help to avoid the use of higher propolis concentrations that could probably affect the quality of honeybee wax and also avoid unnecessary wastage of propolis. Propolis can naturally occur in beeswax to a certain degree, but higher concentrations may be undesirable in some uses of wax, such as in the cosmetic industry where propolis can cause allergy.

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