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Original Article

Exposure to thymol decreased phototactic behaviour in the honeybee (*Apis mellifera*) in laboratory conditions

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Abstract – The effects of the terpenoid thymol were evaluated on the phototactic behaviour of the adult honeybee (*Apis mellifera*) 1 and 24 h after a topical application. The behaviour was quantified under different light intensities by measuring the time spent in the light source area and in areas opposite the source. Stimuli of 200 lx induced positive phototaxis of the bees. Thymol administered at 1 ng/bee had no effect on the phototaxic behaviour while bees that had received 10 or 100 ng thymol 1 h before the test were less attracted by the 200-lx stimulus. The effect of thymol increased when the phototaxic behaviour was tested 24 h after the topical application. However, with a light intensity of 400 lx the dose 10 ng/bee was ineffective and for 600 lx the phototaxic behaviour of the bees was not modified by the exposure to thymol.

thymol / honeybee / phototaxis / laboratory bioassay

1. INTRODUCTION

The search for alternatives to pesticide use, antibiotic treatment of diseased beehives and the fight against *Varroa destructor* is linked to the preservation of the environment but also to the problem of resistance to some molecules. An interest in essential oils has grown as these products could represent a natural alternative to the use of synthetic substances (Waliwitiya et al. 2009; Nerio et al. 2010). In recent years, substances from plants have been tested for their repellent action against parasites, their insecticidal activity (Umpierrez et al. 2011) and also for reducing *Nosema* infection in the bee (Costa et al. 2010). The repellent and insecticidal activities of essential oils are largely due to the presence of monoterpenes and phenolic compounds in particular thymol (Pandey et al. 2009). Thymol is the main phenolic compound derived from *Thymus vulgaris* that has proven to be effective in reducing the development and survival of adult mosquitoes (Pavela et al. 2009; Tabanca et al. 2010) and cockroaches (Phillips et al. 2010). In addition to the insecticidal properties, thymol may also have acaricidal properties against the varroa mite (*Varroa destructor*) (Imdorf et al. 1995). This ectoparasite of the honeybee has developed resistance to synthetic drugs (Milani 1999), and this has led beekeepers to move into new molecules such as thymol. Thymol is used in the form of crystals or commercial formulations (Thymovar®, Apiguard® and ApilifeVar®). Thus, at the end of summer when the hives are treated against the varroa mite bees may be exposed to thymol. In an early study conducted in the laboratory, it was reported that a stream of air containing thymol in doses between 5 and 15 μg/L could kill 100 % of the mites exposed without affecting bee survival (Imdorf et al. 1995).

However, residues were found in honey and wax and a negative effect of thymol-based treat-

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ments on the colony was reported (Floris et al. 2004). Recently, Mondet et al. (2011) observed in laboratory assays that bee behaviour could be modified by the gel formulation Apiguard®. Foragers were repelled by Apiguard® and presented energetic fanning behaviour.

Given the cellular targets of thymol, in addition to its repellent effects, other sublethal effects of this molecule can exist. In the mollusc *Lymnae acuminata*, thymol reduced the levels of serotonin (Singh et al. 1999). Serotonin was found to be involved in the phototactic behaviour of bees (Thamm et al. 2010). Moreover, insect tyramine and octopamine receptors were discussed as molecular targets of essential oil components including thymol (Blenau et al. 2011). It was reported that octopamine influences *period* gene expression and locomotor behaviour in the honeybee (Bloch and Meshi 2007). These authors had suggested that the light perception of bees could be modified by octopamine treatment (Bloch and Meshi 2007). In *Drosophila*, it was shown that thymol is an inhibitor of transient receptor potential-like (TRPL) channels (Parnas et al. 2009). Visual perception of *Drosophila* involves TRPL channels (Cosens and Manning 1969; Landry et al. 2007; Delgado and Bacigalupo 2009) and TRPL expression in *Drosophila melanogaster* peaks at the end of the day (Wijnen et al. 2006). Thus, it appeared relevant to study the visual function of the bees when they were exposed to thymol. To address this question we investigated the phototactic response of bees to light sources of different intensities after a topical application of thymol.

2. MATERIAL AND METHODS

2.1. Bees

The colony and the hive had not received any treatment specially miticide treatment for 1 year before the beginning of the experiment. Adult bees were collected in the morning from an indoor and an outdoor hive from October to December. During this period, bees continued to leave the hive. The bees were transported to the lab in glass vials and immobilized by cooling the vials to 4 °C in ice. The animals were transferred individually in a 5-mL syringe with six ventilation holes. Because responsiveness to visual stimuli is correlated with gustatory responsiveness i.e. bees displaying a high gustatory responsiveness are also sensitive to light (Erber et al. 2006); motivation of bees to sucrose was checked before the phototaxis evaluation. Bees were fed with 10 μL of a 30 % sucrose solution, then starved for 3 h before the test. The syringes were used for retention during feeding and application of thymol on the bee's thorax. They were also used for transporting bees to and from the open-field and for contention during the resting periods at 33 °C after tests that did not exceed 48 h. Mortality was monitored over the 48 h following treatment.

2.2. Thymol exposure

The LD_{50} of thyme essential oil was 8 mg/bee (Albo et al. 2003). Thymol has been shown not to be toxic at the doses tested in the present experiments and the doses tested could be considered as sublethal. In addition, in the present experimental condition, no increase in the longevity of thymol-treated bees could be expected for a short period of 48 h (Costa et al. 2010). One hour before the test, bees received a 1-μL drop of thymol solution on the thorax. Thymol (thymol minimum 99.5 %, Sigma) was first dissolved in acetone then diluted in water. Acetone at 0.1 % was present uniformly in all the solutions used for the topical application. Four experimental groups were conducted in parallel: control group (0.1 % acetone) and thymol groups composed of bees treated at 1, 10 and 100 ng/bee. Each group was composed of six bees. Experiments conducted with 200 lx were repeated three times (18 bees in total for each group) and two separate experiments were performed with 400 and 600 lx (12 bees in total for each group).

2.3. Measurement of phototaxis behaviour

The experiments were conducted at the individual level on bees and allowed precise control of experimental parameters: quantity of thymol delivered to each bee, physiological state and duration of exposure. The walking behaviour of each bee was

measured 1 and 24 h after thymol application. In their natural environment, honeybees are positively gravitaxic and positively phototaxic when they walk along the frames to the exit of the hive. The light intensity at the level of the bottom board was 30 lx inside the hive and 1,200 lx outside the hive. The phototaxis behaviour was analysed in an open field designed to investigate locomotor activity (Lambin et al. 2001). So we modified the protocol previously used in the design to reach the conditions met by bees in a hive. The arena (length, 30 cm; height, 30 cm and depth, 4 cm) was standing vertical. The PVC back of the area was divided into six horizontal levels of 5 cm (level 1: bottom of the device, level 6: top of the device) with each level divided into squares of 5× 5 cm. In the present protocol each bee was introduced in the top left corner of the device. It was illuminated from its right bottom corner where the bee could find the exit (Figure 1a). The light stimuli were produced by two quartz halogen lamps (150 W) with intensity variation. Light intensities of 200, 400 and 600 lx were measured directly in square 1.1 2 cm from the source with a lux meter. The source was 35 cm from the open-field entrance. A computer was placed on the right of the light source on the same table. The observations took place in a cabin with a black curtain kept in the dark. Data were collected for 3 min. The variables assessed were the total length walked, the duration of immobility, the time spent in level 1 and level 6, in the light stimulus area (square 1.1), in the area of the same level but opposite the light stimulus (square 1.6), in the area 25 cm above the source (square 6.1), in the area diagonal to the source (square 5.5) and in the entrance area (square 6.6). The amount of locomotor activity was assessed by time of immobility and the length of the path. These parameters are relevant to investigate potential effects of thymol on motor and arousal functions but

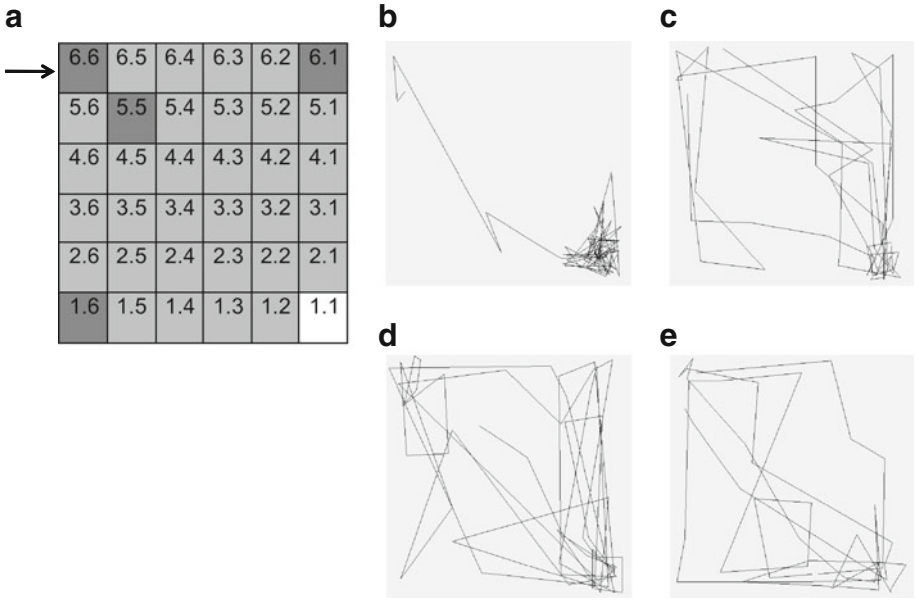

Figure 1. Examples of walking paths of an individual bee tested to the 200-lx stimuli. Bees were introduced in the experimental setup (**a**) used for phototaxis investigation at the top left corner (*arrow*) 1 h after treatment. The bee had received, on the thorax, 1 μl of a 0.1 % acetone solution (**b**) or a thymol solution containing 1 ng (**c**), 10 ng (**d**) or 100 ng (**e**). Recording was performed 1 h after the thoracic application during 3 min. Phototaxis was analysed by the measurement of the time spent in the light source area (*square 1.1 in white*), in the entrance area of the open field (*square 6.6 in dark grey*), in the area vertically above the source (*square 6.1 in dark grey*), in the area horizontal to the source (*square 1.6 in dark grey*) and in the area diagonal to the source (*square 5.5 in dark grey*).

are not suited for investigations on phototaxis. The analysis of the spatial pattern, performed by the measurement of time spent in the squares, can reveal the phototaxic behaviour.

2.4. Data analysis

The same bee was tested only for a single light intensity. Data are presented as mean values ± SEM. Differences among treated groups were assessed by analysis of variance (ANOVA) and Scheffe's post hoc tests. Differences were deemed statistically significant at $P<0.05$. Normality and equal variances of data used for ANOVA were confirmed.

3. RESULTS

3.1. Bee mortality

Mortality was measured for the 48-h period of observation after the beginning of the treatment. Mortality was not significantly different between groups ($Chi^2=5.9$, df=3, $P=0.115$). In the present experimental conditions, the mean percentage of dead bees in each group was 44±27 (control), 48±19 (1 ng/bee), 62±27 (10 ng/bee) and 44±26 (100 ng/bee) after the topical application. ANOVA did not indicate a significant difference between groups ($F=0.812$, dl=3, $P=0.50$). The mortality of bees treated with thymol was comparable from those of control bees within the 48-h period of observation.

3.2. Amount of locomotor activity

A first experiment was conducted with a 200-lx light intensity. When introduced in the vertical open-field, honeybees moved to level 1 and went to square 1.1. The bees first moved with positive gravitaxis from level 6 to level 1 then, they walked to square 1.1. This movement towards the 200-lx light source was a positive phototaxis (Figure 1b). The total length walked and the duration of immobility (Table I) were not significantly different across the treated groups when tested 1 h after treatment (ANOVA, total length: $F=2.205$, df=3, $P=0.09$; immobility duration: $F=0.448$, df=3, $P=0.720$). Once again, when bees were exposed to 400 lx, there was no significant difference in the length walked ($F=1.178$, df=3, $P=0.33$) and the duration of immobility ($F=0.789$, df=3, $P=0.51$). The total length ($F=0.274$, df=3, $P=0.84$) and the duration of immobility ($F=0.625$, df=3, $P=0.60$) were not modified by treatment when bees were tested for a 600-lx light intensity (Table I).

By contrast, 24 h after treatment the distance covered by the bees differed between groups (ANOVA: $F=7.242$, df=3, $P=0.0006$) when bees were tested for the 200-lx stimulus (Table I). A significant difference was revealed between control and 100 ng groups, control and 10 ng groups, 1 ng and 100 ng groups and 1 ng and 10 ng groups, ($P<0.05$ Scheffé pairwise post hoc tests). However, immobility duration was identical under this stimulus condition ($F=1.207$, df=3, $P=0.32$) and for the higher intensity of light (400 lx: $F=1.862$, df=3, $P=0.17$; 600 lx: $F=0.442$, df=3, $P=0.72$; Table I). These results indicated that in low (200 lx), medium (400 lx) and high (600 lx) intensity light conditions, thymol had no effect on locomotor activity 1 h after a topical application. However, 24 h after treatment 10 and 100 ng thymol-treated bees were more active than control bees under the low-intensity stimulus (200 lx).

3.3. Phototaxis

With 200-lx illumination, the 1 h test indicated that the time spent in square 1.1 was significantly different between groups ($F=5.17$, df=3, $P=0.0029$; Figures 1b–e and 2a). Control bees were present in the square 1.1 for a longer period than bees that had received 10 or 100 ng thymol ($P<0.05$ Scheffé pairwise post hoc tests). This time for the 24 h test (Figure 2b), a difference was found for level 6 between groups ($F=13.25$, df=3, $P=0.0001$). Control and 1-ng/bee groups spent less time than the 100-ng/bee group in level 6 ($P<0.05$ Scheffé pairwise post hoc tests). A significant treatment effect was still observed for level 1 ($F=24.777$, df=3, $P=0.0001$). Control bees were still

Table I. Thymol effect on locomotion of bees.

		Immobility (s)		Total length walked (cm)	
		1 h	24 h	1 h	24 h
200 lx	Control	5±7a	0.6±1a	220±58a	385±126a
	1 ng/bee	7.8±20a	3.4±7a	232±78a	350±123a
	10 ng/bee	3.9±7.5a	2.6±4.2a	279±93a	516±62b
	100 ng/bee	3.6±4.4a	1.2±1.6a	228±71a	519±75b
400 lx	Control	29±31a	0.8±1.5a	167±66a	485±240a
	1 ng/bee	29±31a	8.6±9a	178±69a	342±175a
	10 ng/bee	58±55a	28±41a	113±70a	398±172a
	100 ng/bee	42±50a	1.8±2.7a	168±87a	585±208a
600 lx	Control	18±25a	6.8±10a	204±97a	390±200a
	1 ng/bee	18±19a	17±28a	213±96a	411±237a
	10 ng/bee	15±19a	8.3±18a	232±114a	352±254a
	100 ng/bee	29±35a	9.1±15a	195±93a	373±170a

Duration of immobility (s) and the total length (cm) were recorded during the 3-min test. The same bee was tested for a single stimulus intensity (200, 400 or 600 lx), 1 and 24 h after treatment. Mean ± SEM followed by different letters are significantly different at the α=0.05 level (ANOVA followed by Scheffe's test)

more present in this level than bees of the 10 and 100 ng groups (P<0.05 Scheffé pairwise post hoc tests). In addition, a significant difference between groups was still observed for square 1.1 (F=24.831, df=3, P=0.0001). According to Scheffe's tests, control and 1 ng/bee groups spent more time than 10 and 100 ng/bee groups in square 1.1 (P<0.05 Scheffé pairwise post hoc tests).

When the behaviour of the bees was examined 1 h after treatment and with 400 lx (Figure 2c), a significant effect of treatment was still observed (F=4.762, df=3, P=0.008) in square 1.1 with less time spent in this square by the 100 ng/bee group in comparison to the control (P<0.05 Scheffé pairwise post hoc test). Twenty-four hours after the topical application (Figure 2d), between-group differences in time spent in square 1.1 did not reach a significant level (square 1.1: F=1.062, df=3, P=0.39).

Again, for the highest intensity of the stimuli (600 lx), ANOVA indicated that there was no difference between groups when tested 1 h after treatment (square 1.1: F=1.225, df=3, P=0.3125; Figure 2e) and 24 h after treatment (square 1.1: F=0.553, df=3, P=0.65; Figure 2f).

4. DISCUSSION

The search for alternatives to pesticides is related to the preservation of the environment and reducing the impact on non-target insects i.e. the honeybee. The present research aimed to investigate if thymol exposure could have a negative impact on the state of the colony.

Because an increase of bee longevity has been reported for thymol (Costa et al. 2010), we measured the mortality of bees all along the experiment. No significant difference was found between groups in the conditions used. The mortality level was not modified by thymol. However, half of the bees died 48 h after treatment. This relatively high mortality rate can be partly explained by the stress of the different manipulations of bees especially the cooling and the storing of bees individually in a syringe during resting periods.

Here, we analysed the effect of thymol on bee behaviour; we examined the locomotion of bees in laboratory conditions 1 and 24 h after a topical application. It has recently been shown in *Phaenicia sericata* that topically applied

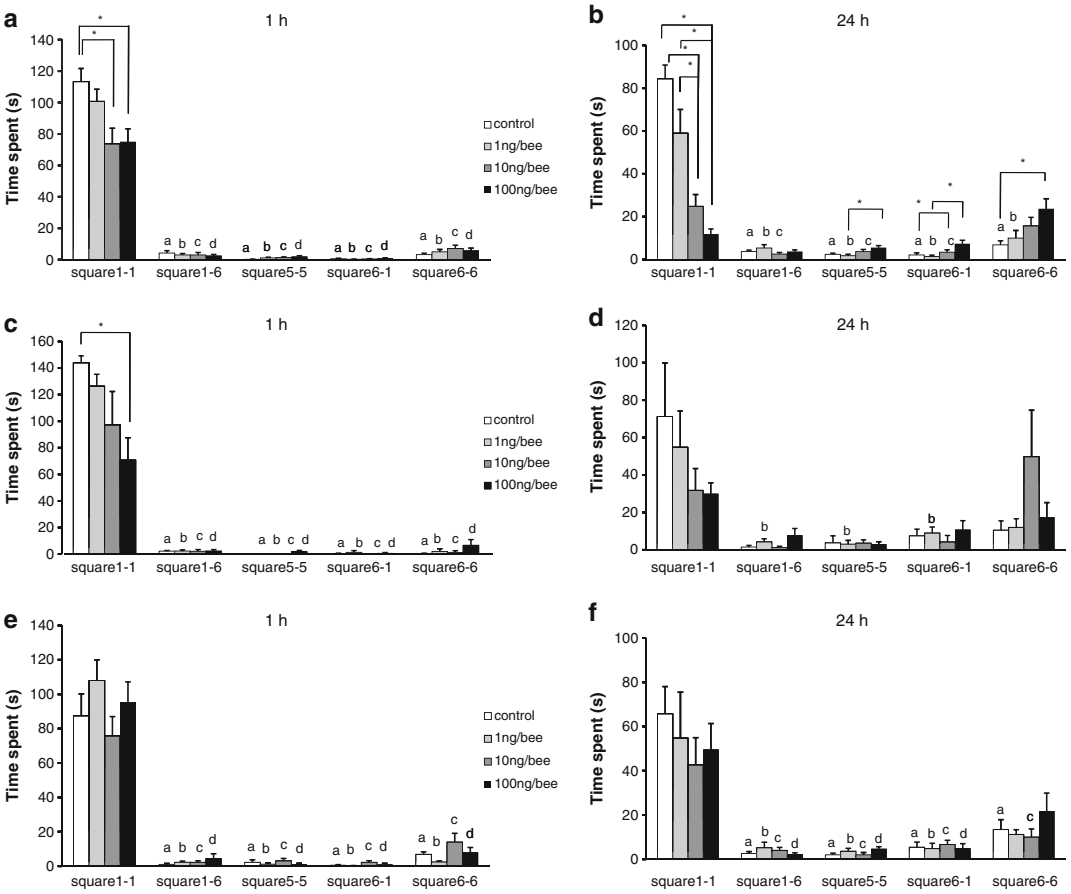

Figure 2. Effect of thymol on the phototaxic behaviour. Bees were tested for different light stimuli: 200 lx (**a**, **b**), 400 lx (**c**, **d**) and 600 lx (**e**, **f**). Tests were undertaken 1 h (**a**, **c**, **e**) and 24 h (**b**, **d**, **e**) after topical application of thymol (1 μl). The time spent in the light source area (*square 1-1*), in the entrance area of the open field (*square 6-6*), in the area vertically above the source (*square 6-1*), in the area horizontal to the source (*square 1-6*) and in the area diagonal to the source (*square 5-5*) is indicated by mean ± SEM from separate experiments performed in sextuplicates. Experiments were repeated three times with 200 lx and two times with 400 and 600 lx. Statistically significant differences between treated groups are indicated by an *asterisk* (ANOVA and Scheffe's post hoc tests). The comparisons to square 1-1 were indicated by different letters for each treated group: *a* for the control group, *b* for the 1 ng/bee group, *c* for the 10 ng/bee group and *d* for the 100 ng/bee group (repeated measures ANOVA, and Scheffe's post hoc tests).

thymol (50 μg) reduced dorsolongitudinal muscles activity and the wings were motionless (Waliwitiya et al. 2010). By contrast, our results indicated that 24 h after a topical application (100 ng/bee), bees were more active. This suggested firstly that in our case thymol had no effect on muscles. Secondly, bees may have been more active because the application of thymol promoted a general arousal similar to that caused by octopamine (Roeder 1999), perhaps suggesting that thymol had targeted octopamine receptors.

Here, effects of treatments were compared for walking parameters, in a device that was illuminated at its bottom right corner. At 1 ng/bee, thymol had no significant effect on locomotion or phototaxis. However, bees exposed to 10 or 100 ng were less attracted than control bees to light stimuli of low intensity.

This strongly suggested that the visual function could be impaired by thymol. Indeed, in addition to $GABA_A$ channels (Priestley et al. 2003), thymol targets TRPL channels. TRP channels were first described in blind *Drosophila* mutants (Cosens and Manning 1969) and TRPL are of particular interest in phototransduction (Wang and Montell 2007). More recently, it was demonstrated in *Drosophila* that thymol inhibits TRPL channels (Parnas et al. 2009). Because thymol-based treatment was proposed for use at the end of summer, we investigated the effect of thymol from October to December, probably on winter bees. The long-lasting effects of thymol on summer bees need to be investigated. Indeed, foragers are known to be strongly positively phototactic (Menzel and Greggers 1985) in comparison to nurses (Ben-Shahar et al. 2003). Moreover, it was found that honeybee responses to treatment with a thymol formulation (Apiguard) change with age (Mondet et al. 2011). So additional data, taking into account the age of bees, seem necessary for investigations into the effect of thymol on the visual function of this insect. Moreover, because octopamine, tyramine and $GABA_A$ receptors are potential targets of thymol in the honeybee (Enan 2005; Priestley et al. 2003; Blenau et al. 2011), it is possible that thymol also affects behaviours that are relevant for division of labor, e.g. olfactory learning and sucrose perception. Indeed, it has been observed that phototactic behaviour correlates with gustatory responsiveness (Erber et al. 2006).

The work provides new insights into the biological and behavioural effects of thymol in the honeybee and will allow beekeepers to improve their technical expertise in the use of this molecule. Specific instructions to minimize exposure to bees should be provided.

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L'exposition au thymol réduit le comportement phototactique des abeilles (*Apis mellifera*) en conditions de laboratoire

Thymol / abeille / phototactisme / essai en laboratoire

Thymolkontakt verringert das phototaktische Verhalten der Honigbiene (*Apis mellifera*) unter Laborbedingungen

Thymol / Honigbiene / Phototaxis / Labor / Bio-Assay

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