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Apis mellifera (Insecta: Hymenoptera) in the target of neonicotinoids: A one-way ticket? Bioinsecticides can be an alternative

Ane C.C. Santos[a], Paulo F. Cristaldo[a], Ana P.A. Araújo[b], Carlisson R. Melo[a], Ana P.S. Lima[c], Emile D.R. Santana[c], Bruna M.S. de Oliveira[a], José W.S. Oliveira[d], Jodnes S. Vieira[d], Arie F. Blank[c], Leandro Bacci[a,c,*]

[a] Programa de Pós-Graduação em Agricultura e Biodiversidade, Universidade Federal de Sergipe, São Cristóvão, SE, Brazil
[b] Departamento de Ecologia, Universidade Federal de Sergipe, São Cristóvão, SE, Brazil
[c] Departamento de Engenharia Agronômica, Universidade Federal de Sergipe, São Cristóvão, SE, Brazil
[d] Departamento de Zootecnia, Universidade Federal de Sergipe, São Cristóvão, SE, Brazil

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ABSTRACT

The recent decline of *Apis mellifera* populations around the world has been subject of intense research due to ecological and economic damages resulting from the loss of pollination services. The intensive use of insecticides from the neonicotinoids group is among the possible causal factors of this decline, including also sub-lethal effects. However, the use of synthetic insecticides has been increased on a global scale in the recent decades. In order to evaluate an alternative to the use of neonicotinoids, this work investigated the effects of a bioinsecticide and its major compound on *A. mellifera* (Apidae: Hymenoptera), one of the main pollinators of crop plants. For this, bees were exposed, by contact and ingestion, to the essential oil of *Cymbopogon martinii* (Poaceae: Poales), to geraniol (major compound) and the insecticide imidacloprid to evaluate the toxicity and behavioral effects as well as the locomotion changes and immune responses of bees treated with these compounds. In general, toxicity was greater through ingestion and the insecticide imidacloprid was more toxic to *A. mellifera* compared to the essential oil and its major compound. The individual and collective behaviors (*i.e.* trophallaxis, grooming, avoidance) as well as the immune responses of bees were not significantly affected by bioinsecticides. However, the locomotion response and flight orientation of the bees were significantly altered by insecticide when administered by ingestion. Our results highlight the potential of *C. martinii* essential oil and its major compound as a possible alternative to mitigate the harmful effects of neonicotinoids on bees.

1. Introduction

The maintenance of biodiversity ensures ecosystem services, which provides a range of benefits for humans (Dirzo et al., 2014). Among these services, insect pollination – mainly carried out by bees (Klein et al., 2007) – represents a crucial service for maintenance of the genetic diversity of wild plants (Knight et al., 2005) and for the world's agricultural productivity (Ricketts et al., 2008). The recent global decline in *Apis mellifera* populations – known as Colony Collapse Disorder (CCD) - is considered threatening because of the huge economic damage from reduced pollination in different crops (Potts et al., 2010). CCD has been attributed to multiple factors that appear to act synergistically, including: loss of natural habitat, incidence of parasites and diseases and intensification of agriculture (Staveley et al., 2014). Although the relative importance of these factors is not yet known, the use of insecticides from the neonicotinoids group has been reported as an important factor, mainly due to their sublethal effects on bees (Pisa et al., 2017; Sánchez-Bayo et al., 2016). However, some recent studies have also showed that minor doses of neonicotinoids present non sublethal effects on honeybees (Byrne et al., 2014; Dively et al., 2015).

Neonicotinoids can contaminate bees directly during the application in the field and, mainly, through the consumption of resources such as pollen and nectar from contaminated plants (*e.g.* oral exposure), since they are systemic pesticides (*i.e.* once absorbed by plants, they are diffused in tissues of bees) (Farooqui, 2013). These insecticides act on arthropods, causing physiological and behavioral effects by directly interfering in the acetylcholine receptors – neurotransmitter receptors responsible for triggering the depolarization of the postsynaptic membranes in the central nervous system (Gbylik-Sikorska et al., 2015). Although the effects of acute lethal toxicity are not always observed,

* Corresponding author at: Departamento de Engenharia Agronômica, Universidade Federal de Sergipe, São Cristóvão, SE, Brazil.
E-mail address: bacci.ufs@gmail.com (L. Bacci).

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chronic sublethal effects (*i.e.* effects from doses that do not cause mortality directly) may contributes to CCD (Henry et al., 2012; Pisa et al., 2017). Among such sublethal effects is reduction of immune response of bees contaminated with neonicotinoids (Brandt et al., 2016), which makes them more susceptible to infection by pathogens (Sánchez-Bayo et al., 2016) [*e.g. Nosema ceranae* (Aufauvre et al., 2012) and *Varroa destructor* (Barron, 2015)]. In addition, other important sublethal effects include the reduction of the learning abilities and memory of the bees. These changes may interfere in the orientation ability, navigation and consequently in the forage efficiency as well as in the return of foragers to their colonies (Henry et al., 2012), culminating in the reduction of population size and colony productivity (Pisa et al., 2017).

A range of studies have been developed to obtain efficient products against insect pests that have reduced negative effects on non-target organisms, such as bees (Furlan et al., 2018). The essential oils from plants (EOs), for example, consist of a complex mixture of volatile components that can interact, triggering different functions in the plant, such as: protection against pathogens, herbivores and/or attraction of pollinator insects and seed dispersers (Bakkali et al., 2008). Therefore, due its bioactivity, the EOs and its constituents isolated – mainly monoterpenes – may consist of potential bioinsecticides. The EOs are considered an alternative to the use of synthetic insecticides to pest control because several desirable characteristics, such as: efficiency in herbivore control, low toxicity to non-target organisms, reduced persistence in the environment and slow induction of insect resistance due the complexity of compounds (Koul et al., 2008).

Although the EOs are natural compounds considering environmentally safe, they are toxic to different insects and they may also cause undesired effects in non-target organisms (Xavier et al., 2015). The EO of *Cymbopogon martinii* plants has as major compound the geraniol, a monoterpene that is also present in the attraction and aggregation pheromone (*i.e.* during foraging) synthetized by bees (Trhlin and Rajchard, 2011). The effects of EO from plants of *Cymbopogon* genus and the compound geraniol have been showed to control a range of insect pest groups (Hernandez-Lambraño et al., 2015; Lima et al., 2013; Tak and Isman, 2016), including sucking insects [EOs: (Costa et al., 2013; Deletre et al., 2015) and geraniol: Baldin et al., 2014; Deletre et al., 2015) for which the neonicotinoids are widely used (Qu et al., 2015). However, the possible effects (*i.e.,* toxicity, behavior and immunity) of this EO on bees has not been investigated.

As *Apis mellifera* are considered the most important pollinators due to their management in different agricultural crops worldwide (Potts et al., 2010), in the present study, we evaluated the toxicity, behavioral, locomotion changes and the immune response of these bees under the effect of the neonicotinoid imidacloprid, the EO of *C. martinii* and its major compound geraniol.

2. Material and methods

2.1. Collection of bees

The individuals of *A. mellifera* used in the bioassays were obtained from four colonies held at Experimental Apiarium of Federal University of Sergipe, São Cristóvão, Sergipe, Brazil (10°55'S, 38°6′W). Forage bees were collected with a flexible nylon funnel (80 cm), which had one end attached to a plastic pot and other inserted at the entrance of the colony, maintaining a slope toward the light. The captured individuals were kept in B.O.D incubator with food supply (50% sucrose solution) for a maximum of 3 h before the experiments.

2.2. Compounds and chemical analysis of essential oil of C. martinii

The EO of *C. martinii* and the compound geraniol (98% of purity) were acquired from Raros Naturals® (Macaíba, Rio Grande do Norte, Brazil) and Sigma-Aldrich® (Steinheim, Germany) companies, respectively. The commercial insecticide used was the neonicotinoid imidacloprid (Bayer CropScience®, São Paulo, SP, Brazil) in form of granules dispersible in water (700 g a.i/kg).

The analysis of the EO components was performed by Gas Chromatography coupled to Mass Spectrometry (GC/MS) and Flame Ionic Detector (GC/MS/FID) using the equipment GCMSQP2010 Ultra (Shimadzu Corporation, Kyoto, Japan) equipped with AOC-20i automatic injector (Shimadzu Corporation, Kyoto, Japan). The separations of components were performed on 30 m, Rtx®-5MS Restek fused-silica capillary column (5% diphenyl–95% dimethylpolysiloxane) with a 0.25 mm internal diameter and 0.25 mm film thickness. Helium 5.0 was used as the carrier gas at a flow rate of 1.0 mL min^{-1}. A one microliter (μL) of the EO sample was injected at a temperature of 280 °C, in a split ratio of 1:30. The oven temperature started with 50 °C (isotherm for 1.5 min), increasing 4 °C min^{-1} until reaching 200 °C and then an increase of 10 °C min^{-1} up to 300 °C, which was maintained for 5 min.

In the GC/MS, the molecules were ionized by electrons with energy of 70 eV and the fragments were analyzed by a quadrupolar system programmed to filter fragments/ions with m/z from 40 to 500 Da, detected by an eletron multiplier. The ionization process for GC/FID was realized by the flame coming from hydrogen gases 5.0 (30 mL min^{-1}) and synthetic air (300 mL min^{-1}). The chemical compounds collected and the electric current generated was amplified and processed in GCPostrun Analysis software (Labsolutions- Shimadzu).

The identification of constituents from EO of *C. martinii* was performed based on the comparison of retention indices of the literature (Adams, 2007). For the retention index, the Van Den Dool and Kratz (1963) equation in relation to a homologous series of *n*-alkanes (nC_9-nC_{31}) was used. Three libraries from the equipment (WILEY8, NIST107 e NIST21) were also used to compare spectra data obtained with those from libraries, using a similarity index of 80%.

2.3. Bioassays

Treatments used in the bioassays were the EO of *C. martinii*, geraniol and imidacloprid. Bioassays were performed in a completely randomized design with four replicates (*i.e.* colonies). It was performed toxicity, behavioral, locomotion, flight orientation and immune response bioassays. Tested bees were submitted to treatments by two exposure routes: contact (by topic application) and ingestion. In the behavioral and locomotion/flight orientation bioassays, the variables were analyzed after 1 and 24 h from exposure of bees to treatments in both exposure routes.

2.3.1. Toxicity

To obtain the dose-mortality curves, it was used initially for each route of exposure, doses which resulted in mortalities between 0% and 100% of individuals. Posteriorly, immediate doses were used to determine the curves. In all bioassays, a replicate consisted of a group composed by eight forager bees previously anesthetized at −8 °C for 2 min to allow the application of treatments. Treated individuals were placed in a Petri dish (9 × 2 cm) covered with filter paper and food supply (50% sucrose solution). Petri dishes were maintained in B.O.D. incubator under controlled conditions (26 ± 2 °C, RH 70 ± 5%, darkness) and mortality observations were performed after 24 h. Preliminary tests indicated that methodology used did not affect the survivorship of bees.

For contact exposure route, 1 μL of treatments were applied in the prothorax for each individual using a 10 μL microsyringer (Hamilton®, Renon, NV, USA). To determine the applied doses (μg/individual), the body mass of 40 individuals were determined using a precise analytical balance (AUW220D, Shimadzu). In all treatments, acetone (Panreac, UV-IR-HPLC-GPC PAI-ACS, 99.9%) was used as solvent. Preliminary tests showed that acetone do not affect the survival and behavior of honeybees.

For ingestion exposure route, each bee was individually placed in a

conical plastic tube (30 mm of length and 9–4 mm of diameter) with its head directed to the smaller diameter of the tube. The bottom of the tube (9 mm) was covered with cotton to prevent the escape of individual. After the anesthetic effect, 10 µL of sucrose solution containing the treatments (EO of *C. martinii,* geraniol and imidacloprid) were offered to tested individual. In the control, only 10 µL of sucrose solution was offered. The sucrose solution consisted of a mixture of polyoxyethylene (20) mono-oleosorbitane (Tween 80%) (1%) as a surfactant and 50% of sucrose. Preliminary tests showed that Tween 80% do not affect the survival and behavior of honeybees. Only bees that totally consumed the solution were evaluated.

2.3.2. Immune response of A. mellifera

A common response of the immune defenses of insects, when subjected to invasion by a parasite, is trigger the encapsulation response. To check if the encapsulation response of *A. mellifera* bees is affected by treatments (EO of *C. martinii*, geraniol and imidacloprid), forager bees treated with LD_{20} of treatments by contact and ingestion were submitted to a stimulus (sterile nylon filament) that simulates the presence of parasites. After the application of LD_{20} (as described in toxicity bioassay), a sterile nylon filament (3 mm length and 0.18 mm ø) was inserted through the beginning of abdomen in a way that approximately 1 mm of the filament remained outside the body wall (Brandt et al., 2016; Wilson-Rich et al., 2008). All nylon filaments were sterilized with 99.8% pure ethanol (Proquímios®).

After implantation, bees were transferred to Petri dish (9 × 2 cm) covered with filter paper and food supply (50% sucrose solution). Petri dishes were conditioned in B.O.D. under controlled conditions (26 ± 2 °C, RH 70 ± 5%, without photoperiod) for 24 h. Then, bees were dissected, and the nylon filaments were mounted on slides. It was performed 20 replicates/treatments *per* colony, totalizing 320 nylon filaments for each exposure routes (*i.e.* 640 nylon filaments). The slides were photographed (camera DS-Fi2 coupled to light microscope Nikon SMZ1270) in both side and the capsule area formed in the nylon filaments were analyzed with aid of NIS Elements software (version 4.5). Immune responses of bees were assessed as proportion of encapsulation area measured by dividing the capsule area formed in the nylon filaments *per* total area of nylon filaments.

2.3.3. Individual and collective behaviors

To evaluate changes in the behaviors of bees submitted to the different treatments, individuals were treated with LD_{20} determined in the toxicity bioassays by contact and ingestion (Table 1).

The individual and collective behaviors of treated bees were evaluated according to methodology adapted by Bacci et al. (2015). The bioassays aimed to evaluate: *(i)* the individual behavior changes caused in the treated individual and *(ii)* behavioral responses of a group of untreated individuals in relation to a treated individual.

Each experimental unit consisted of a Petri dish (9 × 2 cm) containing eight forage bees. Among these individuals, one was randomly removed from the dish and it was submitted to application of one of treatments, being relocated to the untreated group after three minutes. Treated bees were marked with a water-based pigment ink marker with no odor or smell (Uni Posca PC-5M; Mitsubishi Pencil Co. Ltd.®). Previous bioassays showed no effect of marker in the behavior of bees. Behavioral observations started after 30 s of reallocation of treated individual. For each colony, 10 replicates/treatments were performed, totalizing 160 Petri dishes analyzed for each exposure route. Behaviors were recorded for 30 continuous seconds interspersed every 30 s, totalizing 5 min of observation *per* Petri dish and 1600 min of total observation.

All individual behaviors of the treated individual were quantified: allogrooming (self-cleaning), ventilation (*i.e.* beats of wings) and elevation of the abdomen displaying the Nasonov 's gland (present in the last abdominal tergite). Collective behaviors performed by untreated individuals in relation to treated individual were also quantified: antennation, grooming (untreated individual cleaning the treated one), trophallaxis, avoidance (from treated individual by untreated one), attraction and aggression.

2.3.4. Locomotion and flight orientation

To verify the effect of treatments on the locomotion and the flight orientation, an arena (cube, 150 cm × 150 cm × 150 cm) with aluminum frame closed with clear plastic was used. At the upper end of the arena was a led lamp (10 W and 806 lm) with a 45° inclination (light source). At the opposite, each treated bee (or control) was individually conditionate, standing 259.8 cm from the light source.

Tested individual was released in the arena after 1 and 24 h of exposure to treatments. The bioassays to analyze the effect of exposure time were conducted independently. For each exposure time, 40 individuals/colony were analyzed, totalizing 320 individuals.

The behavior of each individual was video-recorded every three seconds for a period of one minute, totalizing 20 observations/individual. The observations consisted in registering the position of the tested bee in the arena considering the three dimensions: position *x* (length), *y* (height) and *z* (depth). The activity of individual (*i.e.* moving or not moving), the number of individuals who reached the light source and the time spent to reach the light source were also recorded.

2.4. Statistical analysis

Data of mortality in the toxicity bioassay in both exposure route (*i.e.* contact and ingestion) were submitted to Probit analysis using PROC PROBIT (Finney, 1971) procedure in the SAS software (SAS Institute Inc, 2002).

Data of individual and collective behaviors (*y-var*) in relation to treatments (*x-var*) were submitted, independently, to Analysis of Variance (ANOVA) (R Development Core Team, 2015).

Table 1
Toxicity by contact and ingestion of essential oil of *Cymbopogon martinii*, geraniol and the insecticide imidacloprid on foragers of *Apis mellifera* bees (µg/bee) after 24 h of exposure. Values were estimated by Probit Analysis. *N* = 30 bees/treatment/exposure route.

Treatment	*N*	LD_{20} (95%CI)	LD_{50} (95%CI)	LD_{90} (95%CI)	Slope	$\chi 2$	*P*
Contact							
Essential oil	793	189 (141–233)	465 (404–528)	1828 (1451–2535)	2.2	4.22	0.23
Geraniol	354	124 (101–147)	290 (253–333)	1066 (876–1361)	2.3	0.75	0.86
Imidacloprid	352	0.072 (0.05–0.095)	0.235 (0.194–0.28)	1.44 (1.08–2.10)	1.6	5.67	0.12
Ingestion							
Essential oil	168	54 (49–58)	73 (69–77)	116 (107–128)	6.4	2.19	0.33
Geraniol	254	18 (15–21)	43 (39–49)	156 (130–196)	2.3	5.94	0.20
Imidacloprid	253	0.038 (0.034–0.042)	0.066 (0.059–0.073)	0.148 (0.123–0.191)	3.6	0.39	0.82

LD_{20} = Lethal dose required to kill 20% of population.
LD_{50} = Lethal dose required to kill 50% of population.
LD_{90} = Lethal dose required to kill 90% of population.
95%CI = The 95% lower and upper confidence intervals.

The variations in the locomotion of individuals (flight orientation) in function of treatments (*x-var*) were also submitted to ANOVA. In these analysis, *y-var* were: the mean distance travelled by tested individuals, the mean proportion of activity (moving and not moving) and the mean time spent by tested individuals to reach the light source. The mean time to reach the light source was previously obtained by Weibull (R Development Core Team, 2015; 'survival' package). In this analysis, it was measure the mean time spent for 10 individuals/treatment reach the light source.

The mean proportion of individuals reaching the light source (*y-var*) in function of treatments (*x-var*) was analyzed by ANOVA. Significant differences among treatments were analyzed using Tukey test with *'multcomp'* package (R Development Core Team, 2015).

Data of immune response were submitted to ANOVA with Normal distribution (R Development Core Team, 2015).

3. Results

3.1. Chemical composition of essential oil of C. martinii

It was identified and quantified nine compounds on EO of *C. martinii*, representing 99.73% of the total composition. Geraniol was the compound with highest concentration (85.09%), followed by neryl acetate (6.01%). The other compounds represented less than 3% of the total composition of EO (Fig. 1).

3.2. Toxicity by two exposure routes: contact and ingestion

The dose of compounds required to kill 50% of *A. mellifera* population varied from 0.066 to 465 μg/bee. The EO of *C. martinii* and its major compound were less toxic to bees than the insecticide imidacloprid. The EO of *C. martinii* and geraniol were approximately 1970–1234 (contact) and 1106-652 (ingestion) times less toxic to *A. mellifera* than imidacloprid in the LD_{50}, respectively. The observed pattern was maintained in lower and higher doses (LD_{20} and LD_{90}) (Table 1). All compounds were more toxic to *A. mellifera* when administered by ingestion than by contact. The EO of *C. martinii*, geraniol and imidacloprid were around 6.4, 6.7 and 3.6 times more toxic by ingestion than contact, respectively (Table 1).

3.3. Immune response: encapsulation area

In general, the immune response of *A. mellifera* forager bees was low, presenting an encapsulation area of less than 25%. In both exposure route (contact and ingestion), the encapsulation area was not significantly affected by treatments (*ANOVA;* contact: $P = 0.08$; ingestion: $P = 0.43$) (Table S1; Fig. S1).

3.4. Individual and collective behaviors

There was no significant difference in the individual and collective behaviors of *A. mellifera* in all treatments (EO of *C. martinii*, geraniol and imidacloprid) (Table S2).

3.5. Locomotion and flight orientation

3.5.1. Distance travelled

In general, the average distance travelled by honey bees was negatively affected by treatments in comparison with control (Table 2). However, such negative effect was attenuated in the bees treated with bioinsecticides after 24 h of the exposure. The greatest limitation in the displacement of *A. mellifera* was observed in the individuals treated with imidacloprid compared with EO of *C. martinii* and its major compound.

The effect of treatments in the displacement of individuals did not differ between the routes of exposure, except for imidacloprid that showed more deleterious effect when applied by ingestion (Table 2).

In the exposure route by contact, treatments significantly reduced the maximum distance covered by bees compared to control after 1 h (Table 2, Fig. 2A). After 24 h, only bees treated with EO of *C. martinii* and imidacloprid showed lower displacement (Table 2, Fig. 2B).

Bees exposed to EO of *C. martinii* and geraniol by ingestion for 1 and 24 h presented the highest displacement among treatments (Table 2, Fig. 2C-D), and was not different from control. Although the EO of *C. martinii* and geraniol reduced the displacement of *A. mellifera* individuals compared to control after 1 h of exposure (Table 2, Fig. 2C), with 24 h bees were not affected by these bioinsecticides (Table 2, Fig. 2D).

3.5.2. Activity of A. mellifera individuals

The proportion of individuals moving or not moving was significantly altered with treatments applied by contact (*ANOVA:* after 1 h: $F_{7;24} = 59.546$, $P < 0.001$ and after 24 h of exposure: $F_{7;24} = 141.88$, $P < 0.001$) and administered by ingestion (*ANOVA:* after 1 h: $F_{7;24} = 53.61$, $P < 0.001$ and after 24 h of exposure: $F_{7;24} = 53.53$, $P < 0.001$).

Bees exposed by contact to EO of *C. martinii* and geraniol after 1 h of application moved most of the time in a similar way to that observed in the control, whereas those treated with imidacloprid showed a significant reduction of the movement (Fig. 3A). However, such a negative effect of imidacloprid disappeared after 24 h of exposure (Fig. 3B). Similar pattern was observed in bees treated by ingestion. However, there was no attenuation of the negative effect of imidacloprid after 24 h of exposure.

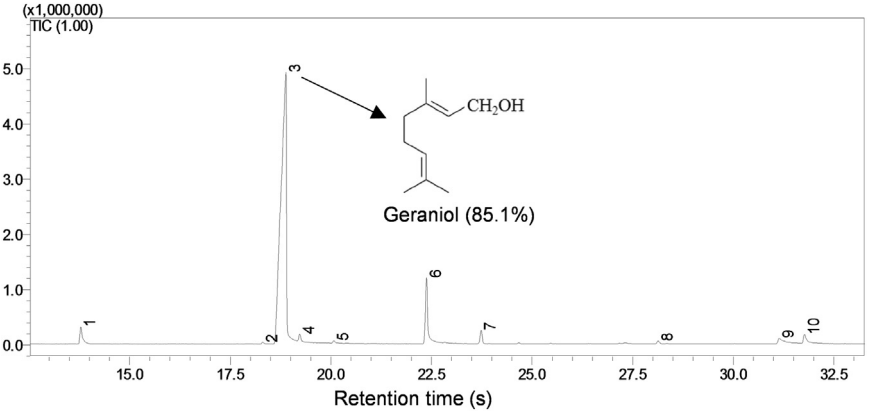

Fig. 1. Chromatogram of the essential oil of *Cymbopogon martinii* analyzed by GC/MS/FID.

Table 2
Maximum distance travelled (mean ± SE) (cm) by *Apis mellifera* bees exposed by contact and ingestion to LD_{20} of essential oil of *Cymbopogon martinii*, geraniol and the insecticide imidacloprid after 1 and 24 h of exposure. *N* = 40 bees/treatment/exposure route/time.

Treatment	Exposure route			
	Contact		Ingestion	
	1 h	24 h	1 h	24 h
Control	236.5 ± 28.3 Aaα	238.8 ± 28.0 Aaα	245.2 ± 19.2 Aaα	216.0 ± 36.0 Abα
Essential oil	161.7 ± 46.3 Baα	187.5 ± 42.6 Baα	196.3 ± 43.1 Baα	219.5 ± 36.1 Aaα
Geraniol	159.1 ± 47.4 Bbα	233.8 ± 25.3 Aaα	193.9 ± 42.6 Baα	216.1 ± 31.8 Aaα
Imidacloprid	124.3 ± 50.5 Bbα	181.3 ± 34.2 Baα	50.6 ± 24.7 Cbβ	116.2 ± 39.3 Baβ

Means followed by same uppercase letters in the column (comparison among treatments), lowercase in the line (comparison among exposure times) and Greek letter in the line for each exposure time (comparison among exposure route) did not differ by Tukey test ($P < 0.05$).

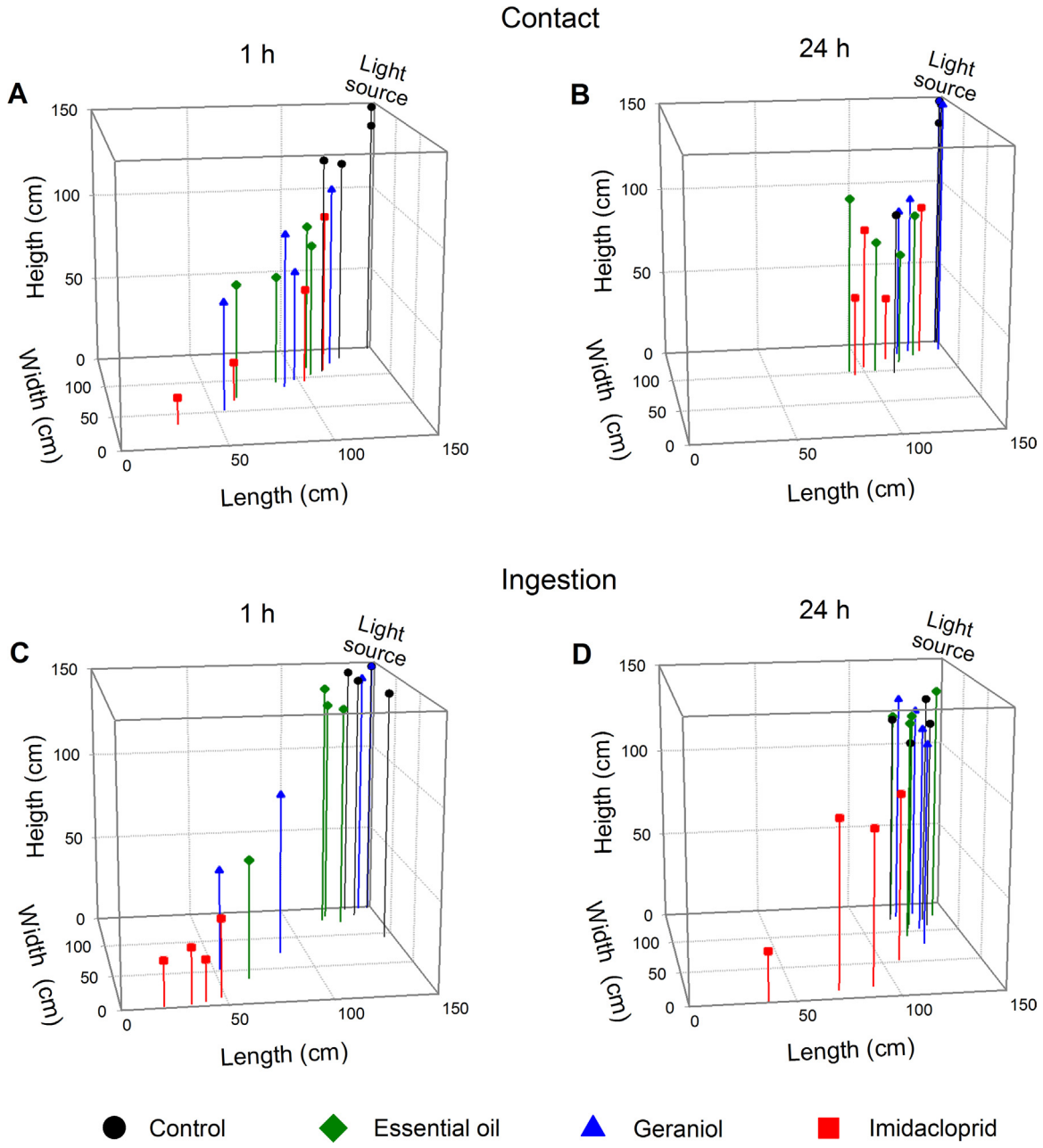

Fig. 2. Mean position reached by *Apis mellifera* forager bees exposed by contact and ingestion to LD_{20} of essential oil of *Cymbopogon martinii*, geraniol and the insecticide imidacloprid after 1 h (A, C) and 24 h (B, D) of exposure. *N* = 30 bees/treatment/exposure route/time.

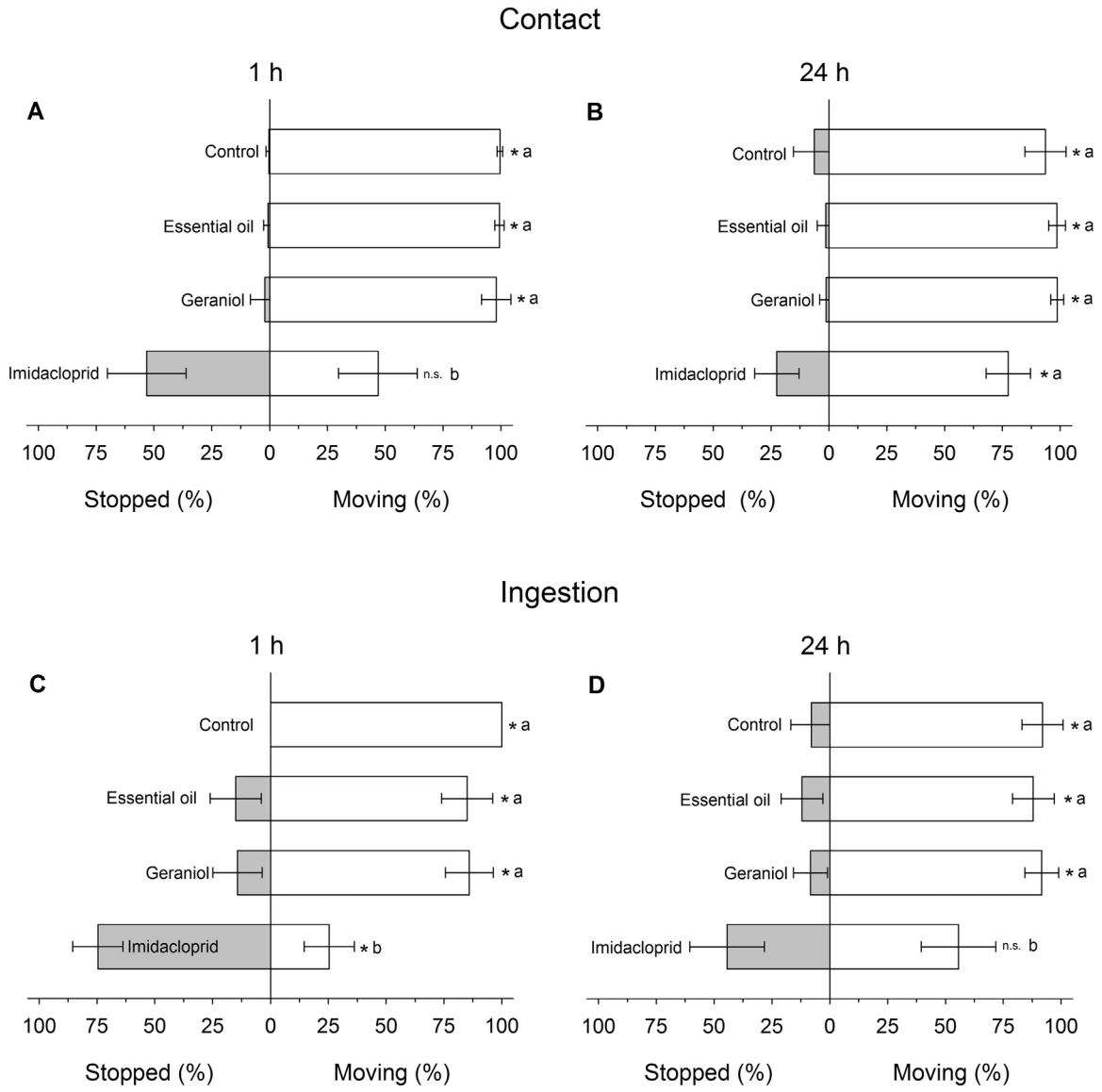

Fig. 3. Mean proportion of time (± S.E.) in which *Apis mellifera* forager bees exposed by contact and ingestion to LD_{20} of essential oil of *Cymbopogon martinii*, geraniol and the insecticide imidacloprid after 1 h (A, C) and 24 h (B, D) of exposure remained moving or not moving. Bars with same letters did not differ statistically from each other by Tukey test ($P < 0.05$). * indicates significant differences in the time that bees remained moving or not moving. $N = 30$ bees/treatment/exposure route/time.

3.5.3. Orientation of A. mellifera individuals

The proportion of individuals reaching at the light source was significantly affected in treatments applied by contact (*ANOVA:* after 1 h: $F_{3;12} = 6.27$, $P = 0.008$ and after 24 h of exposure: $F_{3;12} = 6.28$ and $P = 0.008$) and administered by ingestion (*ANOVA:* after 1 h: $F_{3;12} = 6.69$, $P = 0.006$ and after 24 h of exposure: $F_{3;12} = 15.93$, $P = 0.001$).

All treatments applied by contact (bioinsecticides and imidacloprid), after 1 h of exposure, resulted in a lower proportion of bees reaching in the light source when compared to control (Fig. 4A). However, after 24 h of exposure, only imidacloprid reduced the proportion of individuals reaching in the light source (Fig. 4B).

When administered by ingestion (after 1 and 24 h of exposure), the number of individuals of *A. mellifera* reaching in the light source in the EO of *C. martinii* and geraniol treatments was similar to control (Fig. 4A-B), differing significantly from imidacloprid which bee individuals could not reach the light source (Fig. 4C-D).

3.5.4. Time spent to reach the light source

The mean time spent for 50% of *A. mellifera* individuals reach the light source varied significantly among treatments applied by contact (*ANOVA:* after 1 h: $F_{3;12} = 3.94$, $P = 0.036$ and after 24 h of exposure: $F_{3;12} = 9.18$, $P = 0.036$) and administered by ingestion (*ANOVA:* after 1 h: $F_{3;12} = 3.62$, $P = 0.05$ and after 24 h of exposure: $F_{3;12} = 3.502$, $P = 0.05$).

For individuals treated with imidacloprid by contact after 1 h, the mean time spent to reach the light source was higher than control. Bees treated with EO of *C. martinii* and geraniol showed intermediate times to reach the light source (Fig. 5A). In all other situations, there was no significant difference in the time spent to reach the light source of individuals treated with EO of *C. martinii* and geraniol compared with individuals from control. However, bees treated with imidacloprid were unable to reach the light source (Fig. 5B-D).

4. Discussion

The neonicotinoids are the most used insecticides worldwide, and their consumption have been increased considerable in the last decades on global scale (Pisa et al., 2017). In the present study, our results

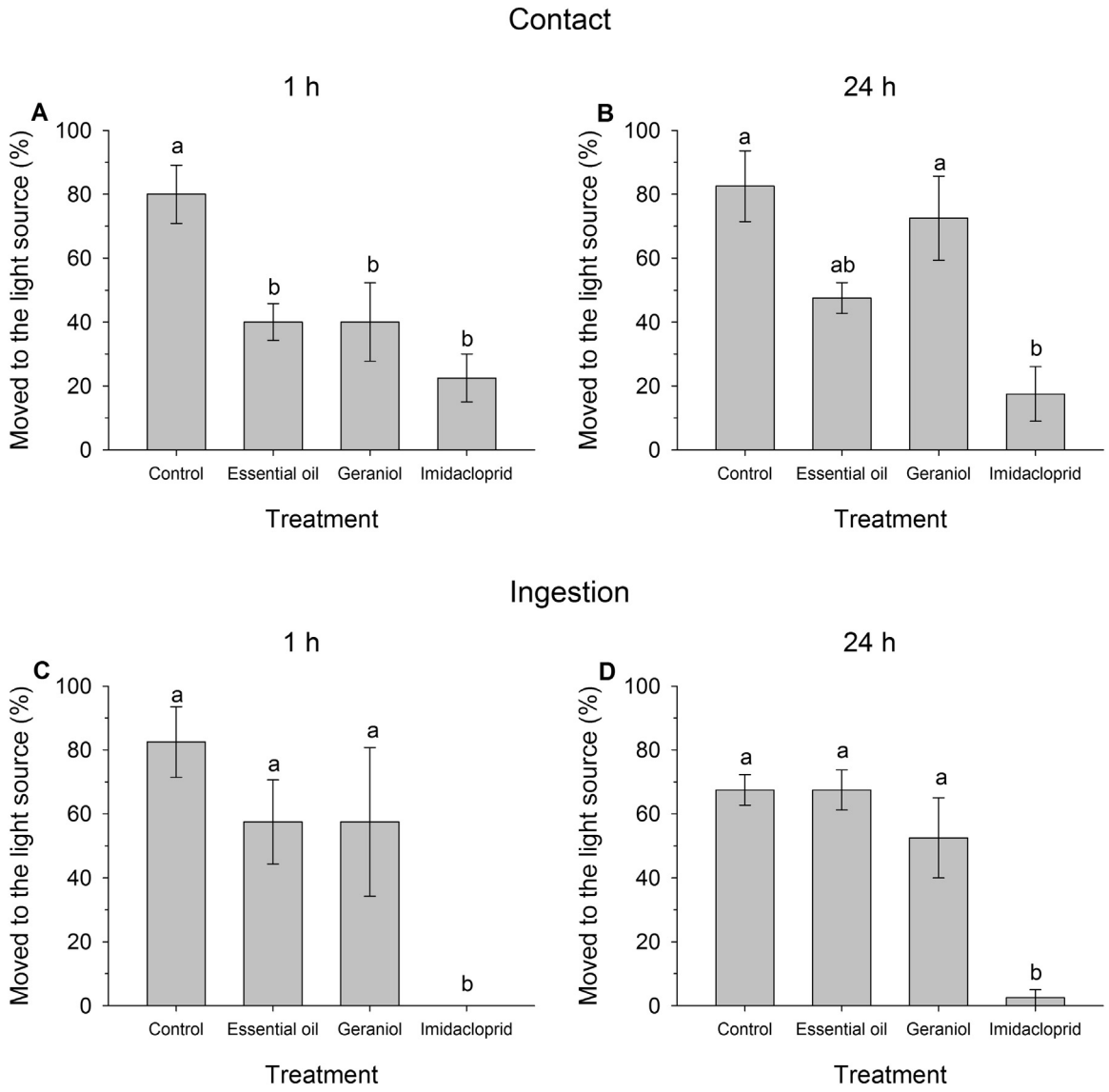

Fig. 4. Mean proportion (± S.E.) of *Apis mellifera* individuals that moved to the light source when treated by contact or ingestion of LD_{20} of essential oil of *Cymbopogon martinii*, geraniol and insecticide imidacloprid after 1 h (A, C) and 24 h (B, D) of exposure. Bars with same letters did not differ statistically from each other by Tukey test ($P < 0.05$). $N = 30$ bees/treatment/exposure route/time.

strongly indicate that bioinsecticides can be a safe alternative to mitigate the effects of neonicotinoids on honey bees. According to the Environmental Protection Agency (EPA), values of lethal doses required to kill 50% of bee population (LD_{50}) higher than 25 µg/bee can be considered as a safe product. Our results showed that LD_{50} of the EO of *C. martinii* and geraniol are greater than 40 µg/bees, which points out as possible non-toxic bioinsecticides for honey bees (United States and Environmental Protection Agency, 2012). In fact, previous studies have been shown that EOs from plants are possible alternatives to pest control since they have toxic effects on insect pests and selective to non-target and beneficial insects (Furlan et al., 2018). The EO of *Cryptocarya alba* and *Carapa guianensis*, for example, did not present toxic effects on *A. mellifera* (Bravo et al., 2017; Xavier et al., 2015).

In general, our results showed that the exposure of *A. mellifera* foragers to EO of *C. martinii* and its major compound (geraniol), in addition to presenting lower toxicity in both routes of exposure, did not present negative effects on the locomotion and orientation of bees under oral exposure. This lack of negative effects of bioinsecticides on honey bees can be explained, in parts, by the efficient metabolic activity of these compounds; since most of monoterpenes are highly metabolized by enzymes such as glutathione S-transferase, esterase and mainly by cytochrome P450-dependent monooxygenase (Stevenson et al., 2017).

On the other hand, the high toxicity and negative effects on locomotion and flight orientation of honey bees treated with imidacloprid can be explained by the neurotoxic action acting as nicotinic acetylcholine receptor agonists (nAChRs) (Matsuda et al., 2001; Wu et al., 2017). The nAChRs mimic the action of the neurotransmitter acetylcholine and prevent degradation by the enzyme acetylcholinesterase, causing the continuous transmission of nerve impulses and subsequent death of the insect. Previous studies, under natural conditions, have also been demonstrated the toxic effects of imidacloprid on *A. mellifera* (*e.g.* Gill et al., 2012; Henry et al., 2012; Pisa et al., 2017). According to Goulson (2013), the contamination of bees depends of the accumulation of the neonicotinoids during the foraging events over the time, including the metabolization and excretion of these contaminants. This could also depend on the rates of ingestion as well as the trophallaxis among individuals and the stock of contaminated products in the colony.

The encapsulation response of *A. mellifera* bees treated with

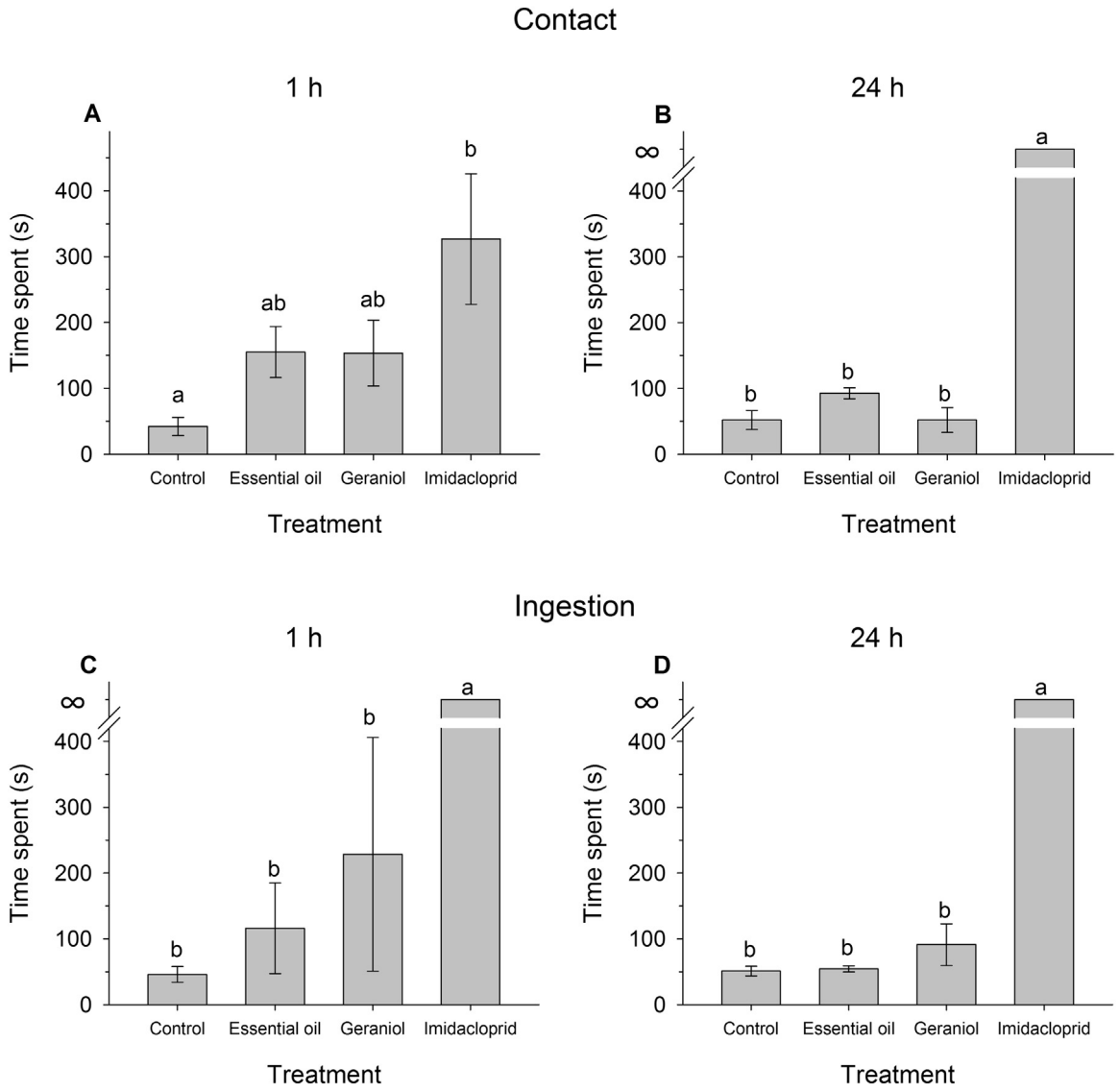

Fig. 5. Mean time (s) spent (± S.E.) for 50% of foragers individuals of *Apis mellifera* bees reach the light source when treated by contact or ingestion of LD_{20} of essential oil of *Cymbopogon martinii*, geraniol and insecticide imidacloprid after 1 h (A, C) and 24 h (B, D) of exposure. Bars with same letters did not differ statistically from each other by Tukey test ($P < 0.05$). $N = 30$ bees/treatment/exposure route/time.

bioinsecticides was not significantly different from honey bees treated with imidacloprid and control. Bees have been reported to present a relatively low set of immune genes in comparison to other insects (Evans et al., 2006). Our results seem to support it, since less than 25% of encapsulated area was observed in all treatments (Fig. S3). A reduction in the immunocompetence of *A. mellifera* bees treated with imidacloprid has already been reported (Brandt et al., 2016; Sánchez-Bayo and Desneux, 2015).

As in other eusocial insects, the defense response can result not only for the humoral response itself, but from behavioral changes among individuals in the colonies. The defense against contaminants can be compensated by the 'social immunity', triggering mainly by prophylaxis behaviors (*e.g.* removal of diseased larvae) and allogrooming (*e.g.* removal of parasites among nestmates), which are modulated mainly by the recognition among nestmates (Evans et al., 2006). In the present study, sublethal doses of bioinsecticides did not changes the individual and collective behaviors of treated bees. Therefore, our results suggest that bee are not able to identify bioinsecticides signals. The absence of change in the individual and collective behavior of forage bees treated indicates that at least their ecological services will be not affected when in contact with bioinsecticides. In fact, the ability of locomotion and orientation of honey bees treated with sub-lethal doses of bioinsecticides were not affected, except for bees exposed by contact with EO of *C. martinii* after one hour. These results suggest that their foraging services would not be affected when exposed to sub-lethal doses of these bioinsecticides.

On the other hand, there was a significant reduction in the total distance travelled, proportion of time moving and locomotion orientation when imidacloprid was administered *via* ingestion, when compared with other treatments. The changes observed in the locomotion and orientation capacity of bees in contact with imidacloprid could, under natural conditions, result in high mortality and failure of foragers to return to their colonies compared with uncontaminated bees, a similar pattern observed by Henry et al. (2012). This effect was even more deleterious when colonies had access to non-familiar and distant foraging sites. Similar effects were also verified for *Bombus terrestris*: a reduction of foraging performance and recruitment, loss of workers and decrease of colony productivity; these effects were intensified when bees were exposed to mixtures of insecticides (see Gill et al., 2012). Any behavior of bees in sense of perceiving and avoiding contaminated sources could reduce these deleterious effects. However, studies have been shown that *A. mellifera*, as well as *B. terrestris*, are

unable to avoid concentrations of the most commonly used neonicotinoids (imidacloprid and thiamethoxan, for example); and in addition, they seem to prefer resource source contaminated instead of non-contaminated resource source (Kessler et al., 2015).

In summary, our results strongly suggest that the use of EO of *C. martinii* and geraniol could contribute to future mitigation actions and conservation of *A. mellifera* colonies. In addition, our study highlights the potential of these bioinsecticides for the management of insect pests since these substances are toxic to insect pests and did not influence the mortality and behavior of *A. mellifera*.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2018.07.048.

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