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ORIGINAL RESEARCH ARTICLE

Effects of neem (*Azadirachta indica*) on honey bee workers and queens, while applied to control *Varroa destructor*

Rebeca González-Gómez[a], Gabriel Otero-Colina[b]*, Juan A. Villanueva-Jiménez[c], Ma. Teresa Santillán-Galicia[b], Cecilia Beatriz Peña-Valdivia[b] and José Antonio Santizo-Rincón[b,†]

[a]*CONACYT El Colegio de la Frontera Sur, Tapachula, México;* [b]*Colegio de Postgraduados Campus Montecillo, Texcoco, México;* [c]*Colegio de Postgraduados Campus Veracruz, Veracruz, México*

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The objective of this study was to determine the effect of neem (*Azadirachta indica*) oil on mortality and development of honey bee worker brood, queen oviposition, colony performance, and *Varroa destructor* mortality. As a hypothesis it was indicated that adequate concentrations of neem oil may control *V. destructor* without affecting bee colonies. Neem oil at concentrations of 0.33–21.1%, with 7.26–464.64 mg l^{-1} azadirachtin, was sprayed on bee (*Apis mellifera*) combs. Their effects on mortality and developmental time of the brood, worker bee response on feeding and capping the larvae, and number of eggs laid by the queen were quantified. A 21.1% oil concentration resulted in 100% egg mortality, but lower concentrations resulted in minimal egg mortality. Larvae that reached the fifth instar were capped and survived the presence of the oil, but when 21.1% was applied, their development was delayed one to two days. With concentrations of 5.3 and 10.6% (116.2 and 232.3 mg l^{-1} of azadirachtin, respectively), queen oviposition was not significantly different from the control, but 10.6% slightly decreased oviposition. The highest *V. destructor* mortality (85%) was proportional to the concentration and number of spray applications. When neem oil was applied to hives, none of the concentrations used decreased bee population, capped worker larvae or the reserves of honey and pollen. However, two queens died after one application of 5.3% and three applications of 10.6%.

Efecto del nim (*Azadirachta indica*) sobre abejas obreras y reinas, cuando se aplica para el control de *Varroa destructor*

El objetivo del estudio fue determinar el efecto de aceite de neem (*Azadirachta indica*) expresado en mortalidad y desarrollo de la cría de abejas obreras, postura de la abeja reina, desempeño de las colonias de abejas y mortalidad de *Varroa destructor*. Como hipótesis se indicó que concentraciones adecuadas de aceite de neem pueden controlar la presencia de *V. destructor* sin alterar las colonias de abejas. Se asperjó aceite de neem sobre panales de abejas (*Apis mellifera*), en concentraciones de 0.33 a 21.1%, con 7.26 a 464.64 mg l^{-1} de azadiractina. Sus efectos se cuantificaron en la mortalidad y tiempo de desarrollo de las crías, en la respuesta de las abejas obreras para alimentar y opercular a las larvas, y en el número de huevos puestos por la abeja reina. La concentración de 21.1% resultó en 100% de mortalidad de huevos, mientras que concentraciones menores resultaron en una mortalidad mínima de huevos. Las larvas que alcanzaron el quinto ínstar fueron operculadas y sobrevivieron a la presencia del aceite de neem, pero cuando se aplicó a 21.1%, su desarrollo se atrasó uno a dos días. Con concentraciones de 5.3 y 10.6% (116.2 y 232.3 mg l^{-1} de azadiractina, respectivamente), el número de huevos puestos por las reinas no fue significativamente diferente respecto al testigo, pero el aceite a 10.6% disminuyó ligeramente el número de huevos. La mortalidad máxima (85%) de *V. destructor* fue proporcional a la concentración y al número de aspersiones. Cuando se aplicó aceite de neem a colmenas, ninguna de las concentraciones disminuyó la población de abejas, la cría operculada de obreras o las reservas de miel y polen. Sin embargo, se perdieron dos abejas reinas luego de una aplicación de 5.3% y tres aplicaciones de 10.6%.

Keywords: azadirachtin; bio-rational control; neem oil; repellency; toxicity

Introduction

The neem tree (*Azadirachta indica*) is recognized for its insecticidal activity. Its recognized properties are toxicity, repellency, feeding inhibition, and alterations in the development of diverse insects, mites, and nematodes. Commercial neem extracts are sold for control of numerous pests (Atawodi & Atawodi, 2009).

The effect of neem as a pesticide is due to a wide variety of substances, of which azadirachtin (AZ) is the most important (Mordue (Luntz) & Blackwell, 1993). Gauvin, Bélanger, Nébié, and Guy (2003) have, however, pointed out that it is the mixture of limonoids that permits complete insecticidal effect. Esparza-Díaz et al. (2010) stated that different extracts can bring together different concentrations of limonoids and accelerate insecticidal effect.

Honey bees can come into contact with neem tree flowers, which attract worker bees to their nectar and, to a lesser degree, their pollen (Crane, Walker, & Day,

*Corresponding author. Email: gotero@colpos.mx

†Deceased.

1984). Moreover, neem extracts are applied for agricultural pest control in crops pollinated by bees, which can take contaminated pollen and nectar to the hive. In this case, bees may become intoxicated when they consume these products (Efrom, Redaelli, Meirelles, & Ourique, 2012; Naumann, Currie, & Isman, 1994; Naumann & Isman, 1996; Xavier et al., 2015).

Neem products have been tested for the control of honey bee (*Apis mellifera*) pests, such as the mites *Varroa destructor* and *Acarapis woodi*, the pathogenic bacterium *Paenibacillus larvae,* and fungus *Ascosphaera apis*, and the protozoan *Nosema apis* (Anjum, Ayaz, Shah, Khan, and Khan (2015); Liu, 1995a, 1995b; Melathopoulos, Winston, Whittington, Smith, Lindberg, Mukai, & Moore, 2000; Melathopoulos, Winston, Whittington, Higo, & Le, 2000; Peng et al., 2000; Schenk, Imdorf, & Fluri, 2001; Whittington, Winston, Melathopoulous, & Higo, 2000). The results of these studies are diverse, but their authors observed that the tested products may harm the bees or alter their development and contaminate the honey.

For control of bee pests with natural products, sub-lethal concentrations have been proposed (Colin, Ciavarella, Otero-Colina, & Belzunces, 1994). That is, applying minimum dosages of active ingredients that effectively control the pests but have fewer undesirable effects, such as bee mortality, contamination of hive products or environmental pollution in general, and have lower cost. Based on the proposals of Colin et al. (1994), González-Gómez, Otero-Colina, Villanueva-Jiménez, Pérez-Amaro, and Soto-Hernández (2006), and González-Gómez, Otero-Colina, Villanueva-Jiménez, Peña-Valdivia, and Santizo-Rincón (2012) demonstrated repellent effects of low concentrations of neem oil on *V. destructor* (hereafter, varroa). These low concentrations did not cause acute toxicity on adult bees, and thus, increase the possibility of using neem. The concentrations of neem oil used by González-Gómez et al. (2006, 2012) and those applied by Peng et al. (2000) (6 and 152 mg l^{-1} AZ) on honey bees and varroa are the basis for new studies.

The objectives of this study were: (1) to determine the effects of neem oil on mortality and development of worker bee brood, queen bee oviposition, varroa mortality and colony performance; (2) to determine concentrations of neem oil effective enough against varroa, and safe enough for honey bees.

Materials and methods

Preparation of neem oil

Ripe neem fruits were collected in an orchard belonging to the Colegio de Postgraduados, Campus Veracruz, in the municipality of Manlio F. Altamirano, Veracruz, Mexico. The pulp (mesocarp) was eliminated manually, and the seed with endocarp was dried for five days at ambient temperature (27 ± 4 °C) with no direct sunlight. With a rotary mill, the endocarp was eliminated and the oil was extracted from the seeds by cold press (1406 kg cm^{-2} pressure; <35 °C) in a stainless steel extractor and stored at −3 °C (González-Gómez et al., 2006, 2012). The AZ concentration was determined in the oil with a modular HPLC system (Schneider & Ermel, 1987).

Effect of neem on mortality and development of worker bees

Thirty-two honey bee colonies installed in Dadant hives were selected at random. From each of these colonies, one comb was selected from the brood chamber; each comb contained worker bee eggs and larvae from first to fifth instar. Specific recognition of each developmental stage of the bee larvae is not a practical enterprise, considering the variation among specimens and their increase in length and weight along successive instars (Winston, 1987). Thus, age groups were formed as a better way to include the successive instars, tentatively recognized by their size: group 1. eggs and first instar larvae; group 2. second and third instar larvae; and group 3. fourth to fifth instar larvae.

Using coordinates, the portions of the comb that contained bees of the above age groups were marked, 90 individuals per portion. To mark the portions, a 5 × 5 cm grid was used in a 42 × 27 cm wooden frame, matching the comb frame size of the Dadant rearing chamber. These areas were selected to observe the effects of neem.

A stock suspension of neem oil plus emulsifier (Tween 20™) and distilled water was prepared (1:1:1, weight). The stock suspension was diluted with water into 1, 2, 4, 8, 16, 32, and 64%, resulting in concentrations of 0.3, 0.7, 1.3, 2.6, 5.3, 10.6, and 21.1% of the original oil. Each concentration of neem was applied to a comb with a Burgerjon's (1956) atomization tower calibrated for application of 1–2 mg cm^{-2}, average 1.7 mg cm^{-2} (Hassan, 1985). This was achieved by spraying 15 ml of the diluted suspension at a pressure of 0.703 kg cm^{-2}, and waiting one minute to allow the droplets to fall onto the honeycombs.

The control consisted of application of distilled water. González-Gómez et al. (2006) demonstrated that none of the concentrations of Tween 20 administered has a toxic or repellent effect on varroa or bees. For this reason, the emulsifier was omitted in the control. Each treatment and the control were replicated four times, giving a total of 32 combs.

After application of neem oil, the combs were placed in their original colonies. On days 0 (immediately before application), 1, 3, 6, 9, and 13, areas selected for observation were photographed to capture the effects of neem, their position recognized by using the grid described above. The number of surviving eggs or larvae of each age group and the number of capped cells were counted. After the rearing cells had been capped, five cells were selected at random from each comb and the cap was carefully cut to withdraw the pupae. They were assessed visually as to whether or not they were alive and their age was estimated in days according to the color changes they undergo, described by Jay (1963) and Rembold, Kremer, and Ulrich (1980).

Effect of neem on queen bee oviposition

The bee colonies used contained queens of *A. m. carnica* (acquired from the company Diproansa S. A. de C. V., Mexico) from the same origin, six months old, and in full egg production. Colony populations were similar (ten spaces between combs full of adult worker bees) and reserves of pollen and honey in at least two combs. A comb with foundation where worker bees could construct cells was placed in each colony. When cells were built, the combs were considered ready for the bioassays. Distilled water (control) was sprayed on the combs, as well as 8, 16 or 32% of the stock suspension of the neem oil. One comb was an experimental unit, and each was replicated five times. Immediately after application, a queen bee was transferred to each comb and both were enclosed in a cage constructed with galvanized wire screen, 5 × 5 wires per square inch (4.191 mm opening) and made to fit a Dadant frame. Through the openings of the screen, worker bees could enter and exit freely from the cage, but the queen bee could not because of her larger size. Each of the devices (comb + queen + cage) was introduced inside the same hive from which the queen and the comb had been extracted. After 24 h the queens were released, the combs withdrawn and the number of eggs deposited was counted.

Effect of neem on bee colony performance and varroa mortality

Thirty-five bee colonies in Dadant hives were selected. All were of the same origin, *A. m. carnica*, with six-month-old queens. They were similar in adult bee and brood population and in honey and pollen reserves. Neem was applied at concentrations of 16 and 32% of the stock suspension, with one (day 0), two (days 0 and 3) or three applications (days 0, 3, and 6) for each concentration, as well as the control, which was distilled water. All treatments and the control were replicated five times. The product was administered on both sides of all combs of each hive, sprayed with a 5 l capacity manual sprayer equipped with a stainless steel nozzle Cat. XR Teejet 80015VS. A side of the comb received approximately 2.5 g of the suspension, equivalent to 50 g per hive, for an average of 1.7 mg cm^{-2}, the same amount of suspension per unit of area used by González-Gómez et al. (2012).

The study was conducted in Montecillo, Municipality of Texcoco, State of México (19° 29′ N and 98° 53′ W, altitude 2250 m). The climate is temperate subhumid [Cb(wo)(w)(i′)g], with summer rains and annual average rainfall and temperature of 645 mm and 15 °C, respectively (García, 2004). Before and after treatment, the following variables were recorded.

Population of worker bees and number of capped pupae

Photographs of both sides of each comb were compared with those taken by Jeffree (1951), representing 150–1500 workers or 125–1800 capped cells. The number of adult bees or offspring was estimated for each comb. The values of each side were added to estimate the population of adults and brood per colony.

Presence of the queen bee

The queen bee was confirmed visually, or it was assumed as present if there were new laid eggs in the hive, recognized by their perpendicular position with respect to the bottom of the cell (Winston, 1987). Presence of the queen was assigned a value of one and her absence had a value of zero.

Food reserves

The area of each comb occupied by honey or pollen was estimated visually and the areas occupied were added and expressed in number of combs or fractions of combs per hive.

Varroa mortality

Before applying the neem treatments, a 45 × 30 cm aluminum sheet impregnated with a thin layer of solid white odorless petroleum jelly was placed on the floor of each hive to capture mites that had died and fallen onto it. Above the aluminum sheet was placed a galvanized metal screen, eight wires per square inch attached to a 6 mm thick wooden frame; the screen prevented the worker bees from removing the dead mites. Aluminum sheets were removed daily and replaced with new ones; fallen mites were counted daily for nine days.

During application of neem treatments, besides the aluminum sheet on the floor of each hive, as described above, the hive entrance was also covered with a galvanized metal screen, 8 × 8 wires per square inch (opening 2.362 mm), attached to a 45 × 10 cm wooden frame, 2 cm thick. The wooden frame had three orifices of 1 cm in diameter on the upper part, to permit passage of the bees, but to interfere in worker bees' ability to remove varroa or dead bees.

After applying the treatments, two strips of Apistan™ (fluvalinate) were inserted into each hive between combs 3 and 4, and 6 and 7, where they remained for two weeks. This acaricide was selected among those commercially available in Mexico due to its proven efficacy (Mutinelli & Baggio, 2004), compared to the lower efficacy of thymol or formic acid-based local formulations (Calderone & Nasr, 1999; Coffey, 2007). Bayvarol™ (flumethrin) was discarded because very high levels of varroa resistance against this acaricide have been detected in Mexico (Rodríguez-Dehaibes, Otero-Colina, Villanueva-Jiménez, and Corcuera, 2011). Fallen mites were counted every week for two weeks. The total varroa population of each hive was estimated by adding dead mites due to the effect of the treatments applied plus the number of mites killed by Apistan. To

determine the effectiveness, the Equation (1) was used (Al-Abbadi & Nazzer, 2003):

$$\text{Efficiency percentage} = (\text{Number dead mites per treatment}/\text{Total population}) \times 100 \quad (1)$$

Experimental design and statistical analysis

The experimental design for all the tests was completely random. The data were subjected to an analysis of variance and means were separated with the Tukey test (SAS Institute, 2015). Percentages were arcsine transformed before the analysis of variance.

Results

Characteristics of the neem oil

The AZ concentration of the crude neem oil was 2200 mg l^{-1}. Neem oil concentrations 1, 2, 4, 8, 16, 32, and 64% corresponded to 7.26, 14.52, 29.04, 58.08, 116.16, 232.32, and 464.64 mg l^{-1} AZ, respectively.

Effect of neem on mortality and development of worker bees

Concentrations of 1–4% neem oil (relative to the stock solution) resulted in larval mortality not significantly different from the control ($p \leq 0.05$) in age group 1 (eggs and first instar larvae). In contrast, larval mortality was significantly higher with concentrations 8 to 32% (Table 1). Twenty-four hours after application of concentration 64%, all the cells of age group 1 were empty. This result may be caused by the hygienic behavior of worker bees (Arathi, Burns, & Spivak, 2000).

The oil at concentrations of 1–32% did not cause significant mortality ($p \leq 0.05$) in age group 2, relative to the control. In contrast, the 64% concentration did cause mortality. In age group 3, none of the treatments was different from the control ($p \leq 0.05$).

During the period of observation, the queen bee laid new eggs in the cells that had been emptied because of the elimination of the eggs or larvae treated with neem oil. The new eggs were not taken into account in the evaluation of larval mortality by neem application. Their presence indicates that the residual effect was of short duration. Brood mortality observed at higher concentrations occurred only with age group 1 (egg and first instar larvae); the queen rapidly replaced them by laying eggs, counteracting the effect of the oil.

In age groups 2 and 3, the number of larvae capped by the worker bees was not significantly different ($p \leq 0.05$) from the control for any of the concentrations of neem oil. A similar effect was observed with the concentrations of 1 and 4% in age group 1. In contrast, concentrations of 8–32% significantly ($p \leq 0.05$) reduced the number of capped larvae. These values include larvae that died at the early stage. Application of 64% neem killed all of the larvae, and so none could be capped (Table 2). However, for all larvae that survived the application of any neem concentration, capping occurred at the expected time in accordance with normal development of the bees (Jay, 1963).

After application of neem oil at concentrations of 1–32%, age of the worker pupae, estimated by their color, on day 9 after oviposition was not significantly different from the control (Tukey, $p \leq 0.05$). In contrast, bees of age groups 1 and 2 treated with the concentration of 64% presented a change in coloration pattern that matched with younger pupae (Jay, 1963; Rembold et al., 1980); this was interpreted as a delay in development. On day 13, the concentrations of 1–32% did not delay development of the worker bee brood, relative to the control (Tukey, $p \leq 0.05$). The 64% concentration, however, in the same period produced a delay in development of pupae. The delay in development varied from a few hours to about 1.5 days (Table 3).

Effect of neem on queen bee oviposition

The number of eggs laid by the queen of the control treatment was not significantly different ($p \leq 0.05$) from that of queens that received neem oil from 1 to 32% (Figure 1). None of the queens died during the evaluation period.

Effect of neem on bee colony performance and varroa mortality

There were no significant differences ($p \leq 0.05$) in the population of worker bees, capped pupae, nor in honey, and pollen reserves when colonies were observed before and after the application of neem oil treatments (Tables 4 and 5). However, two queens were lost, the first in a hive that received an application of 16% neem oil and the other in a hive that received three applications of 32% concentration.

Effectiveness of neem oil at all the tested concentrations, expressed in percentage of varroa mortality, was significantly higher than the control (Table 6). In general, effectiveness was proportional to both the number of applications and the concentration of neem oil. Varroa mortality peaked one day after each application (Figure 2). Peaks were less pronounced after the second and third application. This is associated with the decrease of total varroa population in the hives, resulting from neem applications.

Discussion

Mortality in the bee brood increased with increased concentrations of neem oil. The most susceptible phases were eggs and first instar larvae (age group 1). Similar results were documented by Naumann and Isman (1996) and Peng et al. (2000). Control mortality, especially in age

Table 1. Number of worker bee brood that survived application of increasing concentrations of neem oil (*A. indica*). The bee brood received the respective neem treatments when they were in age groups 1–3, defined below. Numerical values are means ± standard error.

Treatments	0	1	3	6	9	13
Age group 1 (%)	Days after application of neem extract					
0	90 ± 0 a	83.50 ± 1.3 a	80.75 ± 0.7 a	76.00 ± 0.9 a	69.75 ± 1.1 a	65.75 ± 1.6 a
1	90 ± 0 a	81.00 ± 0.4 ab	80.00 ± 0.7 ab	73.50 ± 1.1 ab	71.75 ± 1.3 a	67.75 ± 1.3 a
2	90 ± 0 a	83.00 ± 0.7 a	81.75 ± 0.4 a	75.50 ± 1.5 a	71.50 ± 1.1 a	64.25 ± 2.0 ab
4	90 ± 0 a	79.00 ± 1.4 ab	75.25 ± 0.2 abc	71.75 ± 1.0 abc	69.25 ± 0.2 a	61.25 ± 1.2 ab
8	90 ± 0 a	76.50 ± 2.7 ab	73.50 ± 2.3 bc	68.25 ± 1.1 bc	62.25 ± 1.3 b	58.50 ± 1.1 bc
16	90 ± 0 a	74.00 ± 0.5 bc	70.75 ± 1.7 cd	66.50 ± 2.3 c	61.00 ± 0.9 b	54.25 ± 0.4 cd
32	90 ± 0 a	68.75 ± 1.2 c	66.00 ± 0.9 d	59.00 ± 1.0 d	55.50 ± 0.8 c	51.75 ± 2.1 d
64	90 ± 0 a	0 ± 0 d	0 ± 0 e	0 ± 0 e	0 ± 0 d	0 ± 0 e
Age group 2 (%)	Days after application of neem extract					
0	90 ± 0 a	86.50 ± 0.6 a	86.00 ± 0.4 a	82.75 ± 1.0 a	81.00 ± 1.2 a	79.50 ± 1.0 a
1	90 ± 0 a	85.00 ± 0.4 ab	84.50 ± 0.2 ab	81.75 ± 0.6 a	80.50 ± 0.2 ab	79.50 ± 0.2 a
2	90 ± 0 a	84.00 ± 0.4 ab	83.75 ± 0.4 ab	79.25 ± 1.8 a	78.50 ± 1.5 ab	77.25 ± 1.3 ab
4	90 ± 0 a	84.25 ± 1.6 ab	82.75 ± 1.1 ab	81.00 ± 0.5 a	80.25 ± 0.4 ab	78.25 ± 0.6 a
8	90 ± 0 a	82.75 ± 1.1 ab	82.50 ± 0.9 ab	80.25 ± 0.6 a	78.00 ± 1.0 ab	76.75 ± 1.0 ab
16	90 ± 0 a	85.25 ± 0.4 ab	84.50 ± 0.6 ab	84.00 ± 0.8 a	78.50 ± 1.3 ab	77.25 ± 1.0 ab
32	90 ± 0 a	83.75 ± 1.4 ab	82.75 ± 1.5 ab	80.75 ± 1.4 a	78.00 ± 1.0 ab	73.25 ± 0.8 bc
64	90 ± 0 a	81.75 ± 1.0 b	81.25 ± 0.7 b	80.50 ± 0.5 a	75.75 ± 1.1 b	70.50 ± 0.5 c
Age group 3 (%)	Days after application of neem extract					
0	90 ± 0 a	87.75 ± 0.6 a	85.75 ± 0.2 a	85.25 ± 0.2 a	84.25 ± 0.4 a	84.25 ± 0.4 a
1	90 ± 0 a	86.75 ± 0.4 a	85.25 ± 0.2 a	85.00 ± 0.0 a	83.75 ± 0.2 a	83.75 ± 0.2 a
2	90 ± 0 a	86.00 ± 0.4 a	85.25 ± 0.2 a	85.25 ± 0.2 a	83.75 ± 0.2 a	83.75 ± 0.2 a
4	90 ± 0 a	86.25 ± 0.2 a	85.00 ± 0.0 a	84.50 ± 02 a	83.75 ± 0.2 a	83.75 ± 0.2 a
8	90 ± 0 a	86.25 ± 0.2 a	85.00 ± 0.0 a	84.50 ± 0.2 a	83.75 ± 0.2 a	83.75 ± 0.2 a
16	90 ± 0 a	86.00 ± 0.4 a	84.75 ± 0.2 a	84.50 ± 0.2 a	83.75 ± 0.2 a	83.75 ± 0.2 a
32	90 ± 0 a	85.75 ± 0.4 a	84.75 ± 0.2 a	84.25 ± 0.2 a	83.25 ± 0.4 a	83.25 ± 0.4 a
64	90 ± 0 a	86.00 ± 0.4 a	84.75 ± 0.2 a	84.25 ± 0.2 a	83.25 ± 0.4 a	83.25 ± 0.4 a

Notes: Values in the same column followed by the same letter are not significantly different (Tukey, $p \leq 0.05$). Age group 1: eggs and first instar larvae; age group 2: second and third instar larvae; and age group 3: fourth and fifth instar larvae.

Table 2. Number of worker bee pupae capped after application of neem oil (*A. indica*). The bee brood received the respective treatments of neem oil when they were in age groups 1–3, defined below. Numerical values are means ± standard error.

Treatment (%)	Age group 1	Age group 2	Age group 3
0	69.75 ± 1.11 a	82.75 ± 1.03 a	85.75 ± 0.25 a
1	71.75 ± 1.31 a	81.75 ± 0.63 a	85.25 ± 0.25 a
2	71.50 ± 1.19 a	79.25 ± 1.89 a	85.25 ± 0.25 a
4	69.25 ± 0.25 a	81.00 ± 0.58 a	85.00 ± 0.00 a
8	62.25 ± 1.31 b	80.25 ± 0.63 a	85.00 ± 0.00 a
16	61.00 ± 0.91 b	80.75 ± 0.48 a	84.75 ± 0.25 a
32	55.50 ± 0.87 c	80.75 ± 1.49 a	84.75 ± 0.25 a
64	0 ± 0 d	80.50 ± 0.50 a	84.75 ± 0.25 a

Notes: Values in the same column followed by the same letter are not significantly different (Tukey, $p \leq 0.05$). Age group 1: eggs and first instar larvae; age group 2: second and third instar larvae; and age group 3: fourth and fifth instar larvae.

group 1, was not higher than that observed by Winston (1987) in normal rearing conditions.

Naumann and Isman (1996) observed approximately 75% mortality in bee first instar larvae when 5 µl of a neem-based extract that contained 250 pg azadirachtin was applied topically. However, control mortality was not significantly different from that of fourth instar larvae exposed to 0.25 and 5 ng AZ. In our study, an average of 1.7 mg cm^{-2} neem oil was applied on an area of 0.198 cm^2 at the bottom of a bee cell, and thus the quantity of the product that each bee larvae received was around 0.3604 mg, corresponding to 2.61, 5.23, 10.46, 20.93, 41.86, 83.72, and 167.45 pg AZ per bee, at the respective concentrations. These values are similar to those applied by Naumann and Isman (1996), who observed 100% egg mortality with the highest dosage.

All cells applied with 64% neem oil were empty 24 h after application. It is possible that nurse bees removed the eggs or even ate them, as cannibalism is practiced by worker bees (Winston, 1987). With the information

Table 3. Effect of neem oil at different concentrations on bee brood, age groups 1–3 (see definition of age groups below). The numerical values correspond to the age of the same bees when they had reached the pupa stage (means ± standard error), which was estimated by the color pattern described by Jay (1963) and Rembold et al. (1980). Observations were recorded nine and 13 days after neem treatment.

Treatments	Age group 1	Age group 2	Age group 3
Day 9 (%)			
0	1.375 ± 0.24 a	4.375 ± 0.24 a	6.375 ± 0.24 a
1	1.125 ± 0.13 a	4.125 ± 0.13 a	6.375 ± 0.24 a
2	1.125 ± 0.13 a	4.125 ± 0.13 a	6.250 ± 0.25 a
4	1 ± 0 a	3.750 ± 0.25 a	6.000 ± 0.00 ab
8	1 ± 0 a	3.500 ± 0.29 a	6.125 ± 0.31 ab
16	1 ± 0 a	3.500 ± 0.29 a	6.125 ± 0.13 ab
32	1 ± 0 a	3.500 ± 0.29 a	5.250 ± 0.14 b
64	–	3.250 ± 0.00 b	4.750 ± 0.25 c
Day 13 (%)			
0	3.625 ± 0.24 a	6.625 ± 0.24 a	8.625 ± 0.24 a
1	3.250 ± 0.25 a	6.625 ± 0.24 a	8.625 ± 0.24 a
2	3.250 ± 0.25 a	6.250 ± 0.25 ab	8.500 ± 0.20 ab
4	3.625 ± 0.25 a	6.125 ± 0.13 ab	8.375 ± 0.24 ab
8	3.625 ± 0.24 a	5.875 ± 0.13 ab	8.375 ± 0.24 ab
16	3.375 ± 0.24 a	5.750 ± 0.14 ab	8.000 ± 0.29 ab
32	3.125 ± 0.24 a	5.500 ± 0.20 b	7.750 ± 0.32 ab
64	–	5.375 ± 0.24 b	7.375 ± 0.31 b

Notes: Values in the same column followed by the same letter are not significantly different (Tukey, $p \leq 0.05$). Age group 1: eggs and first instar larvae; age group 2: second and third instar larvae; and age group 3: fourth and fifth instar larvae.

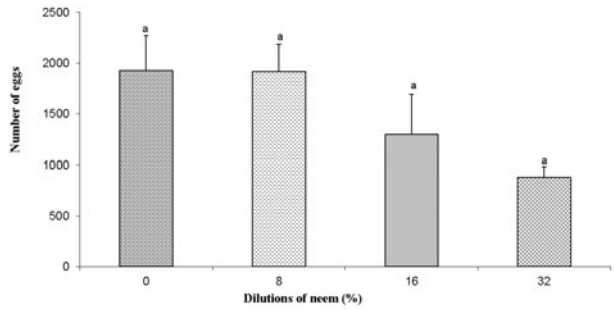

Figure 1. Number of eggs laid in combs in 24 h by queen bees after application of different concentrations of neem oil (*A. indica*).
Notes: Bars marked with the same letter are not statistically different (Tukey, $p \leq 0.05$). Bars represent means; lines above them represent standard errors.

obtained in our study, it is impossible to determine whether cannibalism occurred or workers rejected and eliminated the neem-contaminated eggs.

González-Gómez et al. (2006) determined that 4% neem oil is repellent against varroa, interfering with mite establishment on bee larvae so that they cannot restart their reproductive cycle and finally die, apparently from starvation. In another study, González-Gómez et al. (2012) had similar results with concentrations above 8%. These concentrations of neem oil suggest that a neem-based pesticide or repellent can be developed. The concentration of 64%, or 464.64 mg l^{-1} AZ, should not be used because it can cause egg mortality. Simply by using lower concentrations, this effect can be prevented.

In the study to determine offspring mortality caused by neem oil, with a single application on each comb, a spectrum of ages received the treatment. When it was received at an early age, there was a slight non-significant delay in their development; when it was received at a later larval stage, the delay in their development was more evident and statistically significant. Given that the pupae were alive when they were extracted from their cells, it is debatable whether the delay in their development, estimated at a maximum of two days, actually has negative consequences on the health and productivity of the colony. Similarly, Shawki, Táborský, Kamler, and Kazda (2005) found a delay in the development of worker bee brood when they forced small colonies to forage on rape (*Brassica napus*) treated with a neem-based pesticide.

None of the queen bees died when neem oil was sprayed over the combs and queens were later forced to oviposit on them. No prior studies on the effect of neem on queen oviposition were found. Melathopoulos, Winston, Whittington, Higo, and Le (2000) observed queen mortality resulting from application of neem. However, the data are not comparable because in this part of our study the neem was applied on the combs and not on the queen or the entire hive.

Previous experiments on the effectiveness of neem extracts or purified AZ against varroa have had discouraging results since lethal concentrations were higher for varroa than for bees (Peng et al., 2000), or they resulted in high worker or queen bee mortality (Whittington et al., 2000). Variation on the effect of neem extracts could be associated to the extraction and application method, and also to the plant parts used as a source for the extract (Esparza-Díaz et al., 2010; Imdorf, Bogdanov, Ochoa, & Calderone, 1999; Umpiérrez, Santos, González, & Rossini, 2011). Anjum et al. (2015) evaluated the varroacide efficacy of an extract obtained from neem roots and leaves using methanol, hexane and dichloromethane as solvents. Although these authors did not present the composition of their extract, it is expected to be different from ours, which was a crude oil extracted from neem seeds. They recorded 45% varroa mortality. This is apparently a discouraging result; however such mortality resulted from a single application. Since neem oil has a residual effect no longer than a week (Meisner, Ascher, & Zur, 1983), a single application of neem could cause little effect on varroa mites when they are inside capped cells, and thus, we postulate that repeated applications of a neem extract could result in a more efficient varroacide.

In our work, 85% effectiveness was achieved with three applications of neem oil at 32% of the stock suspension (equivalent to 232.32 mg l^{-1} AZ). This result surpasses levels of effectivity of formic acid treatments,

Table 4. Pre-treatment state of the experimental colonies. Numerical values are means ± standard error.

Treatment	Number of worker bees	Number of capped rearing cells	Presence of queen	Number of combs with honey	Number of combs with pollen
Control	29,700 ± 94.87 a	7780 ± 344.09 a	1 ± 0.00 a	2.1 ± 0.24 a	0.225 ± 0.25 a
16% one application	29,580 ± 203.47 a	7785 ± 253.80 a	1 ± 0.00 a	2.0 ± 0.27 a	0.200 ± 0.03 a
16% two applications	29,520 ± 203.47 a	7890 ± 291.29 a	1 ± 0.00 a	1.9 ± 0.10 a	0.200 ± 0.03 a
16% three applications	29,520 ± 261.53 a	7920 ± 307.35 a	1 ± 0.00 a	1.8 ± 0.12 a	0.175 ± 0.05 a
32% one application	29,520 ± 180.00 a	7905 ± 327.05 a	1 ± 0.00 a	1.8 ± 0.12 a	0.200 ± 0.03 a
32% two applications	29,520 ± 180.00 a	7720 ± 229.73 a	1 ± 0.00 a	2.0 ± 0.27 a	0.200 ± 0.03 a
32% three applications	29,520 ± 203.47 a	7950 ± 280.85 a	1 ± 0.00 a	1.9 ± 0.10 a	0.200 ± 0.03 a

Note: The letter a after values indicates that no significant differences were observed among treatments (Tukey, $p \leq 0.05$).

Table 5. Post-treatment state of the experimental colonies. Numerical values are means ± standard error.

Treatment	Number of worker bees	Number of capped rearing cells	Presence of queen	Number of combs with honey	Number of combs with pollen
Control	29,460 ± 60.00 a	7110 ± 259.04 a	1.0 ± 0.00 a	2.350 ± 0.19 a	0.475 ± 0.03 a
16% one application	29,280 ± 203.47 a	7135 ± 283.35 a	0.8 ± 0.20 a	2.200 ± 0.20 a	0.350 ± 0.07 a
16% two applications	28,980 ± 224.50 a	7280 ± 241.92 a	1.0 ± 0.00 a	2.050 ± 0.50 a	0.325 ± 0.08 a
16% three applications	28,800 ± 300.00 a	7270 ± 266.39 a	1.0 ± 0.00 a	1.925 ± 0.11 a	0.325 ± 0.08 a
32% one application	28,620 ± 180.00 a	7215 ± 249.82 a	1.0 ± 0.00 a	2.100 ± 0.24 a	0.300 ± 0.08 a
32% two applications	29,040 ± 146.97 a	7020 ± 243.46 a	1.0 ± 0.00 a	1.900 ± 0.10 a	0.350 ± 0.07 a
32% three applications	28,620 ± 180.00 a	7130 ± 234.04 a	0.8 ± 0.20 a	1.725 ± 0.179 a	0.225 ± 0.05 a

Note: The letter a after values indicates that no significant differences were observed among treatments (Tukey, $p \leq 0.05$).

Table 6. Effectiveness of treatments for control of *V. destructor* with neem oil evaluated in beehives. Numerical values are means ± standard error.

Treatment	Effectiveness percentage	Total population Mean ± standard error
Control	8.00 ± 0.07 a	5792.6 ± 79.57
16% one application	50.4 ± 0.93 b	5127 ± 97.14
16% two applications	73.0 ± 3.06 c	5093.8 ± 144.35
16% three applications	72.6 ± 0.54 c	5185 ± 47.40
32% one application	68.0 ± 0.84 c	6062.8 ± 66.81
32% two applications	79.6 ± 0.12 d	6171.2 ± 104.99
32% three applications	85.2 ± 0.25 e	6628.4 ± 94.87

Note: Values followed by different letters are significantly different (Tukey, $p \leq 0.05$).

which oscillate around 70% (Feldlaufer, Pettis, Kochansky, & Shimanuki, 1997). Bee mortality attributable to neem oil was negligible, contrasting with 75% observed by Whittington et al. (2000). However, a highly valuable undesirable effect was the death of two queens when neem was applied into entire hives. This may limit the use of neem on bee colonies as tested in this study.

In spite of this problem, we have shown that neem has a higher potential for varroa control than that proposed in previous studies when used directly in the hives. It is necessary to test other dosages and forms of application to reduce the negative effects of neem, such as the death of queen bees in our case. One favorable property of neem is its low toxicity for mammals (Atawodi & Atawodi, 2009).

Peng et al. (2000), who aimed to evaluate the effects of continuous application of neem on bees and varroa, administered it in sugar syrup offered as food; the quantity of neem ingested by the bees accumulated and became very high. Whittington et al. (2000) applied

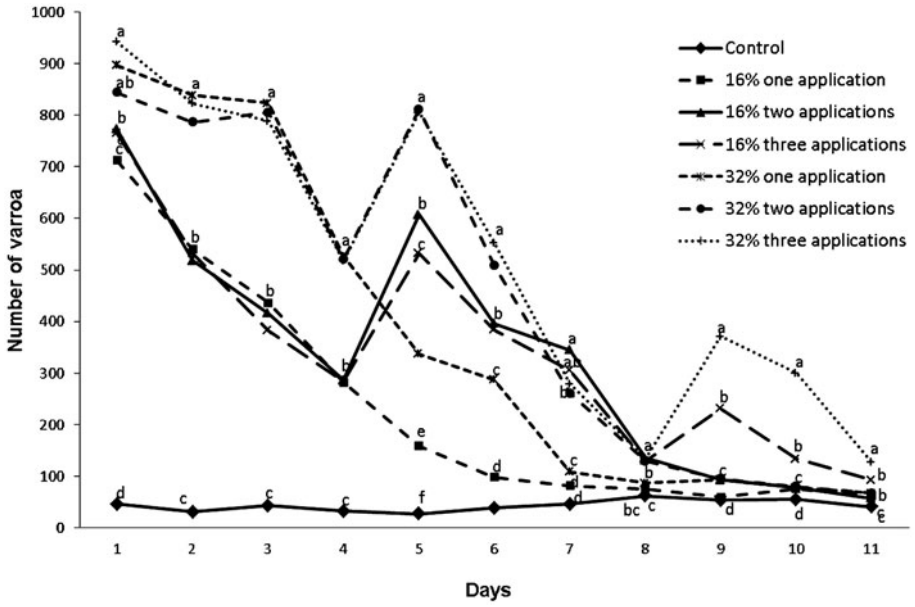

Figure 2. Daily count of fallen varroa mites after application of neem oil treatments.
Notes: Values in different lines on the same day with the same letter are not significantly different (Tukey, $p \leq 0.05$). Neem oil was sprayed one (day 0), two (days 0 and 3) or three times (days 0, 3 and 6).

400 ml of neem suspension per hive on six occasions, literally soaking the bees and the combs at every application. Similarly, Xavier et al. (2015) administered high amounts of a neem-based pesticide (0.7 ml) to individual bee larvae. Perturbation was thus intense and collateral damage, at least partly, could be attributed to the intense handling and the amount of the product applied to the bees. In our study, 50 g of neem oil suspension was applied per hive on a maximum of three occasions, translating into less handling, lower costs and lower mortality of bees, both workers and queens and their offspring.

The proposal of Colin et al. (1994) to use sub-lethal concentrations of plant products for varroa control is sensible, and in this context, the results of our study are promising. It was not possible to determine whether the observed varroa mortality was due to acute toxicity, repellency or other effect inside the hive. However, supported by results of González-Gómez et al. (2006, 2012), we can assume that applying low concentrations of neem interferes in mite behavior in terms of locating bee larvae. In this way, neem impedes mites beginning reproductive cycles and finally causes their death. However, peaks in fallen varroa, which are observed in Figure 2, suggest an immediate, possibly toxic, effect.

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ORCID

Rebeca González-Gómez http://orcid.org/0000-0002-3896-6762

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