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Are the biopesticide neem oil and the predator *Ceraeochrysa claveri* (Navás, 1911) compatible?

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Abstract
Pesticide exposure can result in side effects on natural enemies, making their use impracticable for biological control in agroecosystems. The search for more selective pesticides to minimize the effects on these beneficial organisms is necessary, and among the alternatives, we emphasize the increased use of biopesticides, e.g., neem oil (*Azadirachta indica* A. Juss) (Meliaceae), which is derived from one of the plants among most widely used globally. To determine the compatibility of neem oil with predatory insects in integrated pest management, we evaluated the lethal and sublethal effects on the postembryonic development of the lacewing *Ceraeochrysa claveri* (Neuroptera: Chrysopidae) after neem oil ingestion in the larval instars at a concentration of 0.5%, 1% and 2%. Among the measured biological parameters, we highlighted the accumulated mortality (20.0%, 8.0% and 24.0%; respectively), extension of the larval instars and the significant effects on reproduction (fecundity, fertility and egg viability) were observed for all evaluated doses. In laboratory conditions, the predatory lacewing *C. claveri* and the biopesticide neem oil were not compatible.

Keywords: bioinsecticide, Chrysopidae, predator, selectivity, toxicity

1. Introduction

Currently, the environmental awareness of the world population is growing, and as a result, the consumption of safe and healthy food is desired. To meet this demand, new alternatives to replace synthetic pesticides in growing food have been identified and improved; notably, the use of biopesticides and biological pest control in Integrated Pest Management (IPM). For biological control in pest management to be effective, sustainable and viable, the biopesticide used must be compatible with the populations of the biological control agents that occur in agroecosystems [1, 2, 3].

Among the biopesticides, the interest is high for natural compounds, primarily derived from plants. The natural pesticides obtained from the Indian neem tree (*Azadirachta indica* A. Juss) (Meliaceae) are used worldwide in agricultural crops against arthropod pests. The most popular and easily accessible product is neem oil, which is obtained by cold pressing neem seeds [4, 5, 6]. The predominant active component of neem oil is azadirachtin, which has different activities against insects, such as repellency, antifeedant properties, growth regulation, suppression of reproduction with reductions in fecundity and fertility, and mortality [1, 4, 6, 7, 8, 9].

Recently, the compatibility of neem products with natural enemies have been evaluated, and the neem products were not completely safe for the biological control agents [2, 3, 10]. The neem products did not show selectivity for nontarget organisms, such as green lacewings, either from direct exposure or from indirect exposure with the ingestion of contaminated prey [11, 12, 13, 14, 15, 16, 17, 18, 19]. Green lacewings are polyphagous predators of economically important arthropod pests, such as aphids, thrips, mites, whiteflies, and the eggs and larvae of numerous pest insect species [20, 21]. Among a number of species of green lacewings, *Ceraeochrysa claveri* (Navás, 1911) (Neuroptera: Chrysopidae) is an important predator of phytophagous pests in many Neotropical agroecosystems [22]. Because of a broad prey range, high voracity, high search capacity and short developmental time, *C. claveri* can be considered an efficient biological control agent [20, 21, 23].

Because of its wide distribution in agroecosystems [22], *C. claveri* provided a good biological model to verify the incidence of neem side effects on a predator species. Therefore, our objective was to evaluate the occurrence of lethal and sublethal effects on the postembryonic development of *C. claveri* when exposed to neem oil through oral exposure by feeding on

Poisoned prey during the larval instars. The focus was to investigate the biological effects of neem oil on the development of the insect to adulthood and to verify that some of these effects compromised the ability of this species to be included in a joint biological control program with the use of neem oil.

2. Materials and methods

Insects

The *C. claveri* used in the experiments were provided from a continuously reared laboratory colony from the Laboratory of Insects in the Department of Morphology at the Institute of Biosciences, Brazil. In this colony, the *C. claveri* were maintained in an environmental chamber with controlled conditions (25±1 °C; 70±10% RH; and photoperiod of 12:12 h L:D). The larvae were reared with the eggs of *Diatraea saccharalis* (Lepidoptera: Crambidae), and the adults were fed an artificial diet (1:1 honey: yeast solution).

Biopesticide and treatments

The formulated product used in all of the experiments was neem oil emulsifiable Natuneem® (Natural Rural Ind. e Com. de Produtos Orgânicos e Biológicos Ltda, Araraquara-SP, Brazil; an organic product, certified by BCS ÖKO – Garantie, Doc. Natur - 9009/09.05/7331-BR), which was a pure cold-pressed neem oil extracted from neem seeds that contained 1500 ppm of azadirachtin A as the active ingredient. Three experimental concentrations of 0.5, 1 and 2% (v/v) were used and were prepared separately with the addition of distilled water. We assessed the recommended label rate for use in Brazilian agricultural fields and the concentrations that were previously evaluated by Scudeler and Santos [17].

The fresh egg clusters that were recently deposited by the females of *D. saccharalis* were collected, dipped into each neem oil concentration for 5 s and then air-dried at room temperature for 1 h. For the control group, the egg clusters were dipped into distilled water and then were processed identically to the other groups. The bioassays were performed with the exposure of *C. claveri* larvae to neem oil through the ingestion of the treated eggs. The newly hatched larvae (0-12h) were collected from the stock rearing colony, randomly selected and divided into four experimental groups (n = 30 per group). Each experimental group consisted of 5 replicates per concentration. The larvae were maintained individually in polyethylene boxes (2 cm height x 6 cm diameter) and were tested under identical environmental conditions as described for the rearing of the insects. In the control group, the larvae were fed *ad libitum* the *D. saccharalis* eggs treated with distilled water. For the treated groups (0.5, 1 and 2%), the larvae were fed *ad libitum* the eggs treated with neem oil throughout the larval period until pupation. The egg clusters of *D. saccharalis* were replaced every 3 days because the neem oil compounds were sensitive to ultraviolet light. The experiments were carried out from March to December 2014.

Toxicity assessment

The *C. claveri* larvae were exposed chronically to neem oil via ingestion; thus, we measured the lethal and sublethal effects on the larvae, prepupae, pupae and adults emerged from neem treated larvae. For larval instars, the specimens were checked daily to record the developmental time and mortality. A larva was considered dead when it did not move after being touched with a fine brush. The transition of larval instars was confirmed by the exuviae left after each ecdysis and by the color of the larvae. After larval development, when the larva finished a cocoon, the prepupal period began (Fig. 1a), which continued until the last larval ecdysis inside the cocoon; the larva remained inside the cocoon and transformed into an exarate pupa during the pupation (Fig. 1c). Eventually, a pharate adult emerged from the cocoon. The inability of a larva to spin the cocoon to start the prepupal period (Fig. 1b), the pupal mortality inside the cocoon (Fig. 1d) and the developmental time of the prepupa and the pupa were monitored daily. The adults that emerged from the larvae that survived the treatments were sexed, and the adult sex ratio was calculated as the proportion of females [females/ (males + females)]. To assess the effects on the biological parameters of the adult stage, one freshly emerged female (0-12h) from neem oil treatment was paired with two freshly emerged males from control. The trio of insects was maintained in a polyethylene box (9 cm height x 18 cm diameter) and was provided an artificial diet (1:1 honey: yeast solution) and a cotton wick with distilled water every 5 days until death. Between seventeen and twenty replicates of the adults were used per concentration of neem oil. From these trios of adults, the biological parameters were measured daily and included the preoviposition and the oviposition periods, survival to oviposition, fecundity, fertility and longevity of females. To assess the fecundity, the eggs laid per female were collected daily and were counted throughout oviposition period. The eggs were monitored for egg-hatch as the determinate of the fertility. With the fertility and fecundity data, we calculated the percentage of egg viability: [(fertility/fecundity) X 100].

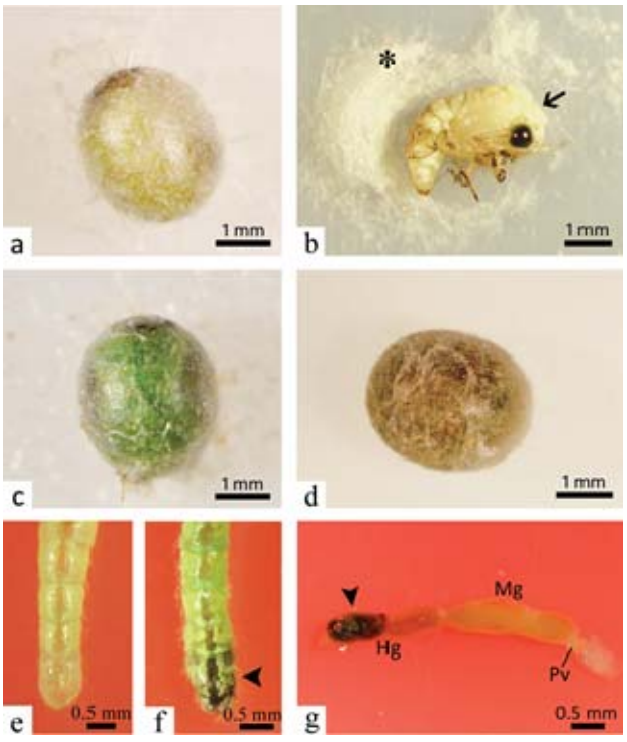

Fig 1: The effects of neem oil on the lifecycle of *Ceraeochrysa claveri*. (**a**) A normal cocoon spun by an untreated larva. The prepupa remains inside the cocoon. (**b**) The exarate pupa (*arrow*) outside a malformed cocoon (*). The larva treated with 0.5% of neem oil did not spin the cocoon in the prepupal period. (**c**) A viable pupa inside the cocoon. (**d**) The dark cocoon indicates pupal mortality inside the cocoon. (**e**) The end of the abdomen of a freshly emerged female from control group. It is possible to observe the green color that is common to the adult *C. claveri*. (**f**) A dark structure (*arrowhead*) is visible inside the abdomen of a freshly emerged female after treatment with neem oil at 2%. (**g**) Part of the alimentary canal of a *C. claveri* adult treated with 2% neem oil. Note the dark structure (*arrowhead*) at the end of the hindgut (Hg), which is the meconium that the adult could not eliminate after emerging from the cocoon. (Pv) proventriculus; (Mg) midgut.

To assist in the description of the change in the prepupae, pupae and adults, the morphology of the normal and the malformed cocoons spun by larvae in the prepupal period, the viable and the nonviable pupae, the abdomen and alimentary canal of female adults emerged from the larvae untreated and treated with neem oil were studied and documented with an Olympus SZX16 stereo microscope with cell^{D} imaging software (Olympus Soft Imaging Solutions GmBH, Münster, Germany) at the Electron Microscopy Center of the Institute of Biosciences, UNESP, Botucatu-SP.

Statistical analyses

The quantitative variables measured following the treatments were examined with analysis of variance, which was complemented with multiple comparison tests. To assess the developmental time of the larvae, prepupae, and pupae, the adult sex ratio and the time of the preoviposition period, the procedure was parametric, which was complemented with the Bonferroni or the Tukey test [24, 25]. The non-parametric test Kruskal-Wallis, complemented with the Dunn test [25], was performed to detect differences in the oviposition period, fecundity, fertility, viability of the eggs and longevity of females among the treatment concentrations of neem oil and the control group.

The study of association relative to the mortality of larvae, the viability of prepupae and pupae and the survival of newly emerged females until the start of oviposition was conducted using the Goodman test that involved contrasts among and within multinomial populations [26, 27]. All tests were performed at a significance level of 5%.

3. Results

For the larval period, significant effects of neem oil were observed on the three larval instars. The larval development time was prolonged in the first instar with the exposure to a dose of 0.5% neem oil; the results were similar for all doses in the second instar but only for the highest dose of neem oil in the third instar (Table 1). In addition to the extended periods of larval development, the neem oil preparations significantly reduced the percentage of survival in the experimental groups because of the accumulated mortality of larvae in the second and third in stars. However, a dose-dependent response was not observed, and both the 0.5% and 2% treatments had the highest percent mortalities of the lacewing larvae (20% and 24%, respectively; Table 2).

Table 1: The effects of neem oil on larval development (mean ± SD) of the different larval stages of *Ceraeochrysa claveri* when fed the eggs of *Diatraea saccharalis* treated with neem oil.

Treatments	**Developmental time (days)**		
	First instar	**Second instar**	**Third instar**
Control	3.813±0.915 aA	4.217±0.889 aB	4.738±0.961 aC
0.5%	4.567±1.644 bA	4.621±1.710 bA	4.925±1.291 abA
1%	4.033±0.986 aA	4.752±1.484 bB	5.000±0.828 abB
2%	4.073±1.593 aA	4.805±1.406 bB	5.246±0.992 bC

The means followed by different letters are significantly different: for lower case letters, the comparison of treatments for the same instar (p<0.05, the Tukey test); and for upper case letters, the comparison of the instars within a fixed treatment (p<0.05, the Bonferroni test). n=150 larvae per concentration.

Table 2: Accumulated mortality (%) of *Ceraeochrysa claveri* larvae of different instars subjected to bioassays with chronic exposure to neem oil through ingestion during larval development.

Treatments	**Accumulated mortality (%)**		
	First instar	**Second instar**	**Third instar**
Control	0	4.7 a	6.0 a
0.5%	0	12.0 ab	20.0 b
1%	0	6.0 a	8.0 a
2%	0	18.0 b	24.0 b

The data followed by different letters, within a column, are significantly different. (P<0.05, the Goodman test). n = 150 larvae per concentration.

The effect of neem oil on the ability of the treated larvae to spin a cocoon was measured in the prepupal period with the formation of viable and nonviable cocoons (Fig. 1a and b). The quantitative effect of neem oil on the prepupal survivorship is shown in Table 3. The toxic effects on the prepupae were more harmful at 0.5% and 1% than at 2% neem oil treatments. Despite the effect on cocoon formation, no significant difference was found in the viability or the pupal developmental time between the neem treatments and the control (Tables 3 and 4; Fig. 1c and d).

Table 3: The effects of neem oil on pupation and pupal viability (%) for the formation of adults of *Ceraeochrysa claveri*, when larvae were fed the eggs of *Diatraea saccharalis* treated with neem oil during larval development.

Treatments	**Prepupa (%)**		**Pupa (%)**	
	Viable	**No cocoon formation**	**Viable**	**Death inside cocoon**
Control	90.0 b	10.0 a	96.3 a	3.7 a
0.5%	78.7 a	21.3 b	94.9 a	5.1 a
1%	78.7 a	21.3 b	94.1 a	5.9 a
2%	82.7 ab	17.3 ab	89.5 a	10.5 a

Percentages followed by different letters, within the same column, are significantly different. (P< 0.05, the Goodman test).

Table 4: Developmental times of the prepupal and pupal stages (mean ± SD) of *Ceraeochrysa claveri* treated with neem oil during larval development.

Treatments	**Developmental** Time (days)	
	Prepupa	**Pupa**
Control	4.700±0.618 aA	9.308±0.582 aB
0.5%	5.036±0.614 cA	9.196±0.534 aB
1%	4.829±0.502 abA	9.234±0.485 aB
2%	4.937±0.691 bcA	9.342±0.610 aB

The means followed by different letters are significantly different: for lower case letters, the comparison of treatments for the same stage (p< 0.05, the Tukey test); and for upper case letters, the comparison of stages within a fixed treatment (p< 0.05, the Bonferroni test).

The sex ratio of the newly emerged *C. claveri* adults was significantly reduced in the 0.5% neem oil group (Table 5). The females emerged from the neem treated larvae were not severely affected in the preoviposition period, except for a

delay in this period in the group treated with 1% neem oil. In the preoviposition period, the low survival percentage of the females that reached oviposition was highlighted, particularly in the 1% and the 2% groups in which just 73.7% and 53% of the females survived, respectively (Table 5). The low survival percentage of the females emerged from the treatments with neem might be related to the difficulty that some females had in the elimination of the meconium from the alimentary canal (Fig. 1e - g). Without the elimination of the meconium, the passage of the alimentary bolus was interrupted, which resulted in death.

Table 5: Adult sex ratio, preoviposition period (d) and adult survival to oviposition (%) of the adults that originated from *Ceraeochrysa claveri* larvae treated with neem oil during the larval stage.

Treatments	Sex ratio[a]	Preoviposition period (days)	Survival until oviposition (%)
Control	0.532±0.019 b[#]	16.92±3.85 a[#]	97.0 c[Δ]
0.5%	0.399±0.083 a	18.11±6.63 ab	91.6 c
1%	0.515±0.062 b	19.13±5.79 b	73.7 b
2%	0.465±0.075 ab	16.92±4.13 a	53.0 a

[#] The means ± SD followed by different letters, within a column, are significantly different ($p < 0.05$, Tukey test).
[Δ] Percentages followed by different letters, within a column, are significantly different ($p < 0.05$, Goodman test).
[a] Adult sex ratio (adults emerged that originated from treated larvae) calculated as the proportion of females = [females/ (males + females)].

Table 6: Oviposition period (d), fecundity, fertility, egg viability (%), and female longevity (d) [median (minimum; maximum value)] of adult of *Ceraeochrysa claveri* fed neem oil during larval development.

Treatments	Oviposition period (days)	Fecundity[a]	Fertility[b]	Egg viability (%)[c]	Female longevity (days)
Control	23.5 (5.0; 67.0) a[#] (18)*	98.5 (13.0; 63.0) b[#] (18)	73.0 (7.0; 130.0) b[#] (18)	73.0 (52.1; 94.9) b[#] (18)	82.5 (60.0; 114.0) b[#] (18)
0.5%	30.0 (1.0; 65.0) ab (20)	32.0 (1.0; 123.0) a (20)	0.0 (0.0; 94.0) a (20)	0.0 (0.0; 85.6) a (20)	70.0 (23.0; 87.0) a (20)
1%	35.0 (14.0; 64.0) b (20)	33.0 (5.0; 107.0) a (20)	0.0 (0.0; 82.0) a (20)	0.0 (0.0; 89.3) a (20)	75.0 (41.0; 101.0) ab (20)
2%	25.0 (5.0; 59.0) a (17)	26.0 (3.0; 135.0) a (17)	0.0 (0.0; 106.0) a (17)	0.0 (0.0; 78.5) a (17)	67.0 (31.0; 90.0) a (17)

[#]Data followed by different letters within columns are significantly different ($p<0.01$, Dunn test).
[a] Fecundity: total number of eggs laid per female throughout the oviposition period.
[b] Fertility: total number of eggs hatched.
[c] Egg viability (%): [(fertility/ fecundity) X 100].
* Number of females evaluated per concentration.

Similar to the preoviposition period, the group treated with 1% neem oil had a prolonged oviposition period (Table 6). The reproductive parameters of fecundity, fertility and egg viability were all significantly lower than those of the control for all concentrations of neem oil (Table 6). Consequently, these sublethal effects on reproduction significantly affected the production of progeny. The adult longevity was also different among the treatment groups, and the females emerged from larvae in the 0.5% and the 2% neem oil treatments had shorter longevity than the females in the control group (Table 6).

4. Discussion

In this study, neem oil induced lethal and sublethal effects on the postembryonic lifecycle of the lacewing *C. claveri*. The assessment of the effects caused by the consumption of poisoned prey clearly reflected an important pathway of *C. claveri* exposure. Because the *C. claveri* larvae are trash-carriers with collected exuviae, dead prey and several pieces of plant material placed on the dorsum [21], in the field, the larvae would be protected from the direct sprays of biopesticides.

Increase the knowledge about the use a specific biopesticide and its effects, primarily the indirect consequence on natural enemies is more relevant and gain interest due to expansion of the use of these pesticides held to be safer to natural enemy's populations [1, 2, 3, 28]. The selectivity of neem oil and the active compound azadirachtin on natural enemies was recently questioned in some studies with green lacewings [11, 12, 14, 15, 16, 17, 18, 19].

In this study, we found that the ingestion of neem oil caused mortality and delay in the development of *C. claveri* larvae, particularly in the second and third instars. Neem oil and the associated compounds affected the larval development of arthropod pests [9, 29, 30, 31], but with widespread use in agroecosystems, neem oil might negatively affect the natural enemies, such as predators. Similar to the results described for *C. claveri* in this study, whether for lacewings or coccinellids, the exposure to neem oil via the intake of prey or with contact caused harmful effects on the larval development of these predators [11, 12, 15, 32, 33, 34]. Because of the repellent and antifeedant effects [6, 8, 9], the higher concentrations of neem most likely resulted in larvae of the first instar that did not feed as much as the larvae in the 0.5% treatment group. Thus, it was possible that only the larvae in the 0.5% group had delayed development in the first instar, whereas the other two instars experienced direct negative effects, particularly at the dose of 2%. The antifeedant and repellent effects could also explain the mortality in the larval stage; the low concentration of neem oil (0.5%) repelled fewer larvae from ingestion of the treated eggs, and therefore these larvae fed on large quantities of eggs and became more susceptible to the harmful effects of the neem. By contrast, the high concentration (2%) of neem oil was strongly repellent to the larvae, but even the small amount of eggs consumed by this group resulted in lethal damage. Although larval development was less susceptible to

a dose of 1%, all the doses had highly detrimental effects on the predator lifecycle. According to Schmutterer [35], the neem products were not completely safe for use with all stages of the natural enemy lifecycle, particularly the larval instars.

Because of the high mortality in the larval stages with exposure to 2% neem oil, when we evaluated the prepupa stage, a higher percentage of cocoons not formed were found for the lower doses (0.5% and 1%) than for the high dose. Scudeler *et al* [36] showed that neem oil intake during the larval stage affected the larval and pupal development of *C. claveri* during the spinning process of the cocoon. These authors demonstrated that the ingestion of neem oil caused morphological and ultrastructural changes in the cocoons and that these specimens were more vulnerable to natural enemies and environmental factors than those that did not ingest neem oil. Although we did not observe significant differences in the viability or the duration of the pupal stage, we believe that this result was because of the manner in which the experiments were conducted, under controlled climatic conditions. Under field conditions, the effects of neem oil on the viability and the development of the pupae might be different from those found in the laboratory evaluations. For many other species that had some form of exposure to neem oil or the components, the harmful effects on the pupal stage were also reported, including for *Coccinella septempunctata* [37], *Harmonia conformis* and *Mallada signatus* [11], *Plodia interpunctella* [29]. And *Spodoptera frugiperda* [30].

The adult females were affected by the treatments with neem oil. According to Rousset [38], in the emergence of adult lacewings, the female reproductive system is not functional and requires some time for the maturation to occur, which is the period that corresponds to preoviposition. Only the group in the 1% treatment showed differences in the preoviposition and the oviposition periods. Ahmad *et al* [31] reported similar effects on the preoviposition period in *Plutella xylostella*. The physiological effects on reproduction might be one of the most important effects following insect exposure to neem [1, 6, 9, 35].

Although the viability of the pupae was high, few females that emerged reached the oviposition period at the larger doses of neem. We observed a dark spot that formed inside the abdomen, a common occurrence in the females treated with neem oil. With an analysis of the internal morphology of these females, we found a dark structure at the end of the hindgut; the females emerged from the cocoon with this structure, which was not be eliminated before reaching adulthood. According to Canard and Principi [39], this structure is the meconium, which is a small, ovoid and compact mass composed of the wastes that were accumulated by the larva during trophic activities. The meconium must be evacuated through the anus for the adult to become active and to begin to feed. Because the ingestion of neem oil affected the midgut epithelium in the larvae and pupae of *C. claveri* [17, 18], we believe these changes influenced meconium formation and prevented its elimination, resulting in shorter lives of these insects and the inability to reproduce.

For the reproductive parameters that we examined, neem had harmful effects on the adult females. A decrease in oviposition, fertility and egg viability led to a reduction in the offspring produced by *C. claveri*, which compromised the growth potential of the population. Interference in the oogenesis and vitellogenesis were the most common alteration reported in the females with inhibited ovarian development and a decrease in female reproduction [13, 40, 41, 42, 43, 44].

Indeed, the pursuit of new strategies for crop protection is necessary, and we cannot neglect the effects of biopesticides on nontarget arthropods. Our results showed that the predator *C. claveri* was affected indirectly by neem oil through the ingestion of poisoned prey. These indirect effects were reflected in all stages of the lifecycle and would likely inhibit the establishment of this natural enemy and prevent an overall success with biological control when attempting to integrate this predator with the use of the neem oil biopesticide. The exposure of the immature stages to neem oil did not provide safety for this predator from the biopesticide, and all stages of the lifecycle exhibited side effects, which were effects that could endanger the population of this species in an agroecosystem and would prevent synergistic control.

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