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Sublethal effects of acetamiprid on native honey bee subspecies (*Apis mellifera*) in Cameroon

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Abstract
Toxic effects of acetamiprid on *Apis mellifera* were tested in the laboratory. Prepared solutions of commercial formulation (4.8 ng/µl, 10.65ng/µl, 21.42ng/µl, 51.52ng/µl, 113.4ng/µl) dispersed in honey syrup and tape water, were administered orally and applied topically on the thorax. Results show that acetamiprid is toxic to *A. mellifera*. Symptoms of neurotoxicity and the first cases of mortality appear 15 minutes after the ingestion of high concentrations and 30 minutes after the topical application of acetamiprid, and the mortality increases according to the concentration and time. LC50 values obtained after 24h are 5.6ng/µl for the topical application and 4.70 ng/µl for the oral route. Our results suggest a particular vulnerability of honey bee behavior to sublethal doses of acetamiprid. Overall, our results are valuable not only in evaluating the toxicity of acetamiprid onto honey bees but also in highlighting the proper application of pesticides for sustainable protection of bees and the environment.

Keywords: *Apis mellifera*, acetamiprid, insecticide, neonicotinoids, toxicity

1. Introduction
To meet the demand for food of the overgrowing population of the world, increasing agricultural productivity has become a priority and crop protection an obligation. Moreover, the use of agrochemical products such as neonicotinoids to protect crops against plant pests and pathogens is a particularly acute concern, not only in terms of economic impacts but also in terms of food resources, especially to fight hunger throughout the world [1, 2]. Therefore, insecticides are applied to a variety of crops to control a wide spectrum of pests [2]. While pest insects are the main targets of manufactured insecticides, non-target organisms such as pollinators may come under their attack [2, 3]. The adverse impact that broad-spectrum insecticides have on non-target beneficial insects is widely known to be a major cause of pollinator decline in cultivated areas [4, 5, 6, 7, 8, 9].

Bees, including honey bees, solitary bees, and bumblebees, are the prominent and economically most important group of pollinators worldwide; 35% of the world food crop production depends on them [10, 3], accounting for an annual value of 153 billion Euros [11]. Therefore, there is a great concern about the decline of the population of honey bee (*Apis mellifera*) in several parts of the world mainly due to the improper application of insecticides [9]. Honey bees and wild bees are of particular interest because they come to contact with various pollutants during their foraging activity on flowers. The drastic effect of pesticides in general and insecticides, in particular, is not limited to the killing of non-target organisms but is related to the abnormal behavior and function they induce [2, 12].

Information on the toxicity of insecticide doses used in the crops in Cameroon on non-target organisms such as honey bee is very important as the country is going for next-generation agriculture. Therefore, the overall aim of the present research is to investigate the toxicity of acetamiprid, commonly used as an insecticide in Cameroon onto honey bee workers. We investigated the effects of different application doses of this insecticide on *A. mellifera* mortality by two exposure methods: topical application and oral feeding at laboratory conditions. To our knowledge, this is the first report on the toxicity of insecticides on honey bee *A. mellifera* in Cameroon and widely in central African countries.

2. Materials and Methods
2.1.Materials
The laboratory works were done from April to September 2018. Animal material consisted of individuals of honey bee caught from the hives of the Apidology Unit of the University of Ngaoundéré. The bees were 30 minutes later introduced into experimental cages each covered with a mesh of 01mm from both sides (Figure 1). The insecticide tested was acetamiprid, which is an insecticide of the neonicotinoid family. It is sold in solid form with a mass concentration of 200g/kg under the commercial name OPTIMAL.

Fig 1: Experimental cages (10 x 8.5 x 6 cm) containing bees

Since their discovery in the late 1980s, neonicotinoid pesticides have become the most widely used class of insecticides worldwide, with large-scale applications

ranging from plant protection, veterinary products, and biocides to invertebrate pest control in fish farming [13]. In this study, we focused our bioassay on acetamiprid (E)-N1-[(6-chloro-3-pyridyl)methyl]-N2-cyano-N1-methyl one of the most commonly misused neonicotinoid in crops protection in Adamaoua Region (Cameroon). Acetamiprid is known to be an ovicidal, larvicidal, and adulticidal, meaning it works at all stages of insect development.

2.2. Methods

2.2.1. Experimental procedures

Laboratory experiments were carried out with *A. mellifera*. Adult workers were obtained from the hives of the Apidology Unit of the University of Ngaoundéré.

Foraging workers were collected as explained by Iwasa *et al*. [14]. Briefly, hives were exposed to smoke twice for 30–60 s before collection. Worker honey bees were collected by shaking from the top super or the front of the hives into a clean and large plastic container. The container was covered with a solid lid, and 30 min. Later, transported to the laboratory. The bees were thereafter kept in experimental cages (figure 1) in groups of 20 at 25±2 °C with 65±5%RH, and fed with honey syrup.

Acetamiprid was dissolved into a mixture of honey and tape water (syrup) to obtain the contaminated stock solution. The control stock solution was the syrup of honey without acetamiprid for the ingestion route. Acetamiprid was used at doses 4.8 ng/µl, 10.65ng/µl, 21.42ng/µl, 51.52ng/µl, 113.4ng/µl. The peasant stock solution (113.4 ng/µl) is the dilution made by peasant according to what they usually do and use to spread their crops.

For topical application, stock solutions were the mixture of acetamiprid and tape water, each concentration corresponding to one stock solution and the last being the peasant stock solution.

2.2.2. Exposure assessment

We had 3 treatments: treatment 1 consisted of tape water; treatment 2 syrup doses of acetamiprid in honey and treatment 3 peasant doses. Each treatment consisted of 3 cages of bees. All cages containing bees were stored at 25 ± 2°C. The test was repeated 3 times, each time renewing the bees according to the method 214 of OCDE [15] and that of 95 of [16] Commission d'Essaie Biologique [15].

The acute toxicity of acetamiprid was evaluated on foragers by two methods, oral administration through spiked syrup and topical application at controlled laboratory conditions. The treatment of each concentration was composed of three replicates. Bees fed with honey solution only and those who received the application of tape water only onto their thorax were used as a control for indirect and direct routes respectively. The mortality was controlled after 1h, 4h, and 24h.

2.2.3.Topical application

For the topical application technique, doses were applied onto the thorax of the bees using a micropipette. Care was taken not to spread the dose on the neck or wing hinges. Treated bees were transferred to the cages, each cage forming a replication with three replicates for each stock concentration. The treatments were kept at 25±2°C, 65±5% RH and observations of the behavior of treated bees started as soon as they are in the cage. The bees were observed from the thirst minute to 24h for mortality. Bees who did not respond to mechanical stimuli were scored as dead. The mechanical stimuli were applied by touching the body of the bees upon each evaluation, using a thin paintbrush.

2.2.4.Oral administration

For the oral administration bioassay, before the tests, bees were first starved for 2 hours at 25 ± 2°C, to facilitate the phenomenon of trophallaxis (mouth-to-mouth exchange of food) and to induce the same level of appetite [2]. Each lot of bee is fed with 200 µl (10µl per worker) of honey diluted with drilling water with 5 increasing concentrations of acetamiprid (stock solution) dissolved in water, for the test treatments, and honey diluted with water for the control.

2.2.5. Data analysis

The percentages of mortality were calculated for each treatment in both bioassay methods and corrected using Abott's equation [17]. Means and standard error (SE) were determined from three independent replicates of each treatment. The log dose-response curves were used for the determination of LC50, LD50, and IC50 values for the insect bioassay according to probit analysis [18]. The confidence limits of 95% were determined by least-squares regression analysis. The analysis of variance (ANOVA) was used to evaluate differences between groups. A difference was considered statistically significant when $P \leq 0.05$.

3. Results

3.1. Results

3.1.1Toxicity symptoms of acetamiprid on honey bee

From direct observations of the behavior of the honey bees in cages after being contaminated, they showed obvious symptoms of poisoning, such as shaking and tremors, uncoordinated and uncontrolled movements, staggering, inability to take up a correct position of the body, and prolonged frenetic movement of the legs and rotation when in the supine position. The observed neurotoxicity symptoms increased with the concentration and time for the two routes. First cases of mortality were observed 15 minutes after the ingestion of the highest concentration (113.4ng/µl) and 30 minutes after the topical application of the lowest. For the peasant dose, these neurotoxicity symptoms occurred 20 minutes after the contact and 5 minutes after the ingestion of the contaminated solution. For the peasant does, for the direct contact, more than $2/4^{th}$ (16/20) died the first hour and the entire bees (20) died the second hour of the experiment. Through ingestion, all the bees (20) died in the first hour of the experiment.

The results of the direct contact toxicity are shown in Figure 2. It appears from this figure that the mortality increases according to the concentration used and the maximum mortality is reached 3 h after topical application where 100% mortality occurred at a concentration of 113.4 ng/µl. In contrast, the lowest concentration (4.8 ng/µl) resulted in the lowest mortality (31.65%). The difference is significant ($p < 0.05$) between the means of the different concentrations (Figure 3). There is a relationship between the dose of acetamiprid administered by contact and the observed mortality. Moreover, it appears from the same figure that the more the concentration is higher, most quicker the mortality occurs. The maximum of the mortality is reached 4h after the topical application of the highest concentration (113.4ng/µl) of the product.

Our results are not in line with those of Laurino *et al*. [1] who

found that acetamiprid and Thiacloprid showed no mortality in the direct tests even 72 h from test initiation.

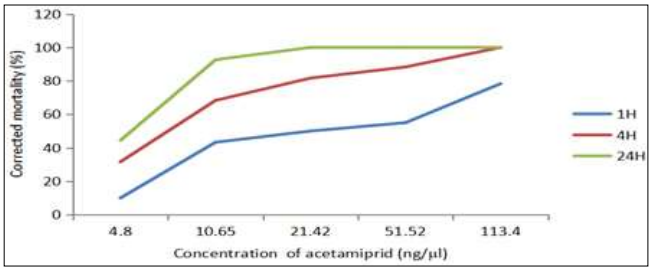

Fig 2: Concentration-mortality relationship of acetamiprid through direct contact on bees

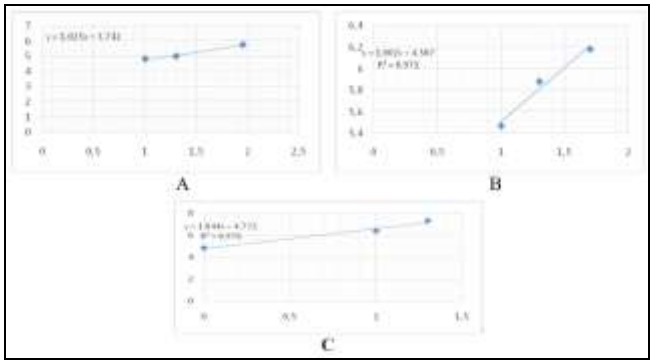

Fig 3: Regression line probit transformation of concentration after 1 (A), 4 (B) and 24 (C) hours of topical application of acetamiprid on bees

3.1.2. Sensitivity of bees

The results of the indirect acute toxicity for these insecticides are shown in Figure 4. The mortality increases with the concentration administered. Indeed, there is a direct relationship between the administered concentration and the observed mortality. The maximum mortality is reached after 1 h if 113.4ng/µl of acetamiprid is administered. There is a significant difference between the means of different concentrations ($p < 0.05$). There is a strong relationship between the dose of acetamiprid administered by contact and the observed mortality (Figure 5).

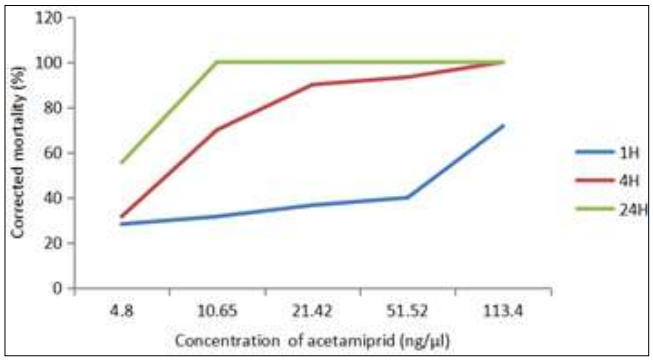

Fig 4: Concentration-mortality relationship of acetamiprid through indirect contact on bees

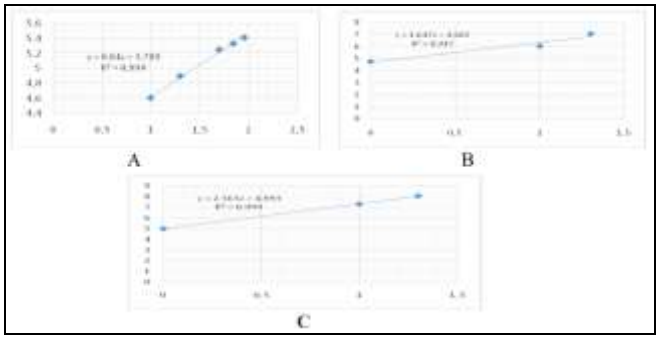

Fig 5: Regression line probit transformation of acetamiprid concentrations after 1 (A), 4 (B) and 24 (C) hours of ingestion

Determination of LC50 of acetamiprid on honey bees through oral and topical application

After topical application and ingestion of acetamiprid, mortality is monitored for 24h bees being observed from their cages. The LC50 values are determined from the regression lines obtained by probit transformations of the corrected percentages and decimal log transformations of the administered concentrations. The values are grouped in Table 1. The LC50 values of acetamiprid obtained after 1 h, 4 h, and 24 h are respectively 20.99ng/µl, 7.41ng/µl, and 5.26ng/µl for topical toxicity, and 43.19ng/µl, 6.79ng/µl, and 4.70ng/µl oral ingestion.

Table 1: CL_{50} of acetamiprid on honey bees

	CL50 (ng/ul/bee)		
	Acetamiprid		
Hours	1h	4h	24h
Contact	20.99[a]	7.41[a]	5.26[a]
Ingestion	43.19[b]	6.79[a]	4.70[a]

It appears from this table that there is no significant difference between LC50 from ingestion and topical application after 24h ($p < 0.05$).

4. Discussion

Observed symptoms induced by acetamiprid on honey bees

Poisoning symptoms similar to those observed in the trials had already been reported by [19, 20, 21] and [22] for various neonicotinoid insecticides. Moreover, the memory and communication abilities of bees could be impaired as shown by [7, 21, 22].

Test results presented in this paper are in line with those reported in the literature even if most concern Imidacloprid on *A. m. mellifera* and *A. m. caucasica* by [23, 24, 25, 26, 27, 28, 29, 30, 1]. These poisoning symptoms linked to the concentration and the route of contamination.

4.1.Honey bee toxicity bioassays through direct and indirect contamination

Apis mellifera bees are susceptible to acetamiprid. Mortality increases as the concentration used increases and the higher the concentration, the faster death occurs. The maximum mortality is reached after 3 hours of treatment at

113.4 ng/µl through the direct and indirect application. Similar findings were done by [31] with thiamethoxam where 100% mortality was generated at 26.01ng/µl/bee after 24 hours with *A. m. intermissa*. [32] obtained 100% of death at 290 ng/µl/bee with thunderous after 24 hours. Our results are not in line with those of [1] who found that acetamiprid and Thiacloprid showed no mortality in the indirect tests even 72 h from test initiation. Moreover, the higher the concentration is, the quicker the mortality. Therefore, the difference in the sensitivity of bees may vary with the speed of action of the products [33]. made the same observations with deltamethrin at 239,50ng/µl/bee where he found maximum mortality after 24 hours.

4.2. LC50 through oral and topical bioassays of acetamiprid on *Apis mellifera*

Laboratory bioassays were conducted to determine the oral and contact toxicity of acetamiprid to *A. mellifera* bees. The LC50 values obtained at 1 h, 4 h, and 24 h are respectively 20.99ng/µl, 7.41ng/µl, and 5.26ng/µl for the topical toxicity, and 43.19ng/µl, 6.79ng/µl, and 4.70ng/µl orally. Analysis of variance confirms that there is no significant difference between oral and topical LC50 for acetamiprid after 24 hours [23]. found that imidacloprid is more toxic when ingested than topically. Meanwhile [31], found a value of 12.29ng/µl orally and 26.01ng/µl per contact on *A. m. intermissa* after 24 h with thiamethoxam. Besides [33], reports an LD50 of 109.72ng/ab through topical application and 239.50ng/bee through ingestion with deltamethrin. Similarly, the toxicity of organophosphates, such as chlorpyrifos, appears 4 times higher through contact than the oral route [34]. At the highest dose, direct contact of the nicotinoid on bees killed quicker than via ingestion [33]. reported that the product ingested passes through the detoxification organs, the intestine and Malpighi tubes, before being distributed throughout the body. On the other hand, the product applied to the thorax passes through the cuticle and waxy tubules and the distribution in the body occurs directly, more particularly in most lipophilic zones. Moreover, it is known that acetamiprid is a neonicotinoid insecticide that works by antagonizing the nicotine acetylcholine receptors in the neural pathways. This causes interruption of brain signals throughout the insect's body.

5. Conclusion

Insecticides can induce more or less serious effects on neural functions that can lead to an impairment of behavior and physiological functions. The mechanisms by which insecticides elicit their effects are not restricted to the exclusive interaction between the active substance and the molecular target responsible for the insecticidal action. Time appears as an important factor in insecticide toxicity. The route and the mode of exposure (acute, subchronic or chronic) play a particularly determining role in nature and the intensity of the effects induced, and are often involved in differential effects elicited by a given substance. The potential means to decrease the side effects of pesticides in the beneficial organisms, particularly the honey bees and pollinators, are of great concern. The acetamiprid tested in this study is one of the most used and misused in the Adamaoua Region. The study, carried out, demonstrated that this insecticide is characterized in *A. mellifera* by the appearance of the symptoms of neurotoxicity such as disordered and fast movements, convulsions followed by tremors, and the mortality occurring after 15 minutes through ingestion and 5 minutes through topical application of high concentration. Similarly, in *A. mellifera*, there is a directly proportional relationship between the administered concentration of acetamiprid and observed mortality and then between mortality and time. Also, the study found that acetamiprid, widely used by farmers, is toxic and that there is no significant difference between topical and oral toxicity 24 hours after being contaminated. These results confirm the danger associated with this insecticide and should not be used during the flowering period of the plants to limit the risk of bee poisoning. More importantly, it would be wise to go for integrated pest management for sustainable management of our environment.

6. Acknowledgment

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