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Effect of Protein Supplementation in the Bee *Apis mellifera* L. Exposed to the Agrochemical Fipronil

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Abstract

Inadequate quantity and quality of proteins in honey bee diet can cause weakening of their colonies and damage their resistance to agrochemical contamination, such as fipronil, which is highly toxic to bees. Thus, we tested the hypothesis if protein supplementation would improve longevity and locomotion of honeybees exposed to fipronil pesticide. Colonies of *Apis mellifera* Africanized were distributed into Control Group without protein supplementation and Supplemented Group with 25% crude protein provided as a paste form at 100 g per week. After four weeks, frames with sealed brood were removed and kept in an incubator until the emergence of worker bees, which were marked, returned to their hives and recaptured six days later to measure protein concentration in the hemolymph. The bee population development was measured by evaluating frames containing the queen's oviposition from each colony. Also, nursing bees were recaptured exposed by contact to fipronil $LD_{50\%}$ (0.009 ± 0.003 µg/bee), and the longevity and motor activity were measured. The results showed that the bee swarms protein supplementation promoted a significant increase in the sealed brood area. However, it did not promote changes in the protein content of the hemolymph. Protein supplementation of bee swarms did not influence the survival of bees exposed to fipronil in the locomotion tests; however, fipronil was toxic to bees and promoted changes in the locomotion of bees.

Introduction

Despite the importance of *Apis* bees, there has been a worldwide decline in the populations of these insects in recent years. Such decline is attributed to climatic factors, use of agrochemicals, diseases, parasites, and habitat and food (e.g. nectar and pollen) reduction, all of which directly impair the maintenance of colonies (Goulson et al., 2015; Wood et al., 2020).

Among these factors, the use of agrochemicals in monocultures contributes to selecting pests that are resistant to the active ingredients used, culminating in the use of more and more toxic products, which are highly harmful to the environment and the pollinators (Johnson et al., 2010).

When bees collect resources (i.e., nectar and/or pollen) that contain high doses of agrochemicals, acute contamination of bees can occur, which causes their mortality over a short period. However, when applied in low doses, considered sublethal, it is often transported to the colony by the bees together with the collected resources, which may compromise the viability of breeding and the maintenance of colonies (Villa et al., 2000; Long & Krupke, 2016). The contamination of colonies by agrochemicals reduces the longevity (Colin et al., 2004; Pettis et al., 2004; Desneux et al., 2007) and affects the vitality of the colony (Belien et al., 2009), amongst other behavioral and physiological changes that impair its survival (Holdera et al., 2018).

One of the agrochemicals harmful to honeybees is fipronil, which belongs to the phenylpirazole group and targets

the receptor of γ-aminobutyric acid as an antagonist. This agrochemical is used worldwide as a pesticide in agricultural and veterinary practices (Kairo et al., 2017). Commonly used on seeds, this systemic insecticide is absorbed by the growing plant and distributed through its tissues, including the flowers (Nauen & Jeschke, 2011). Thus, bees are exposed to residual levels of this insecticide when they collect nectar and pollen from the treated crops (Chauzat et al., 2011). Currently, neonicotinoids and fipronil represent one-third of the global insecticide market (Simon-Delso et al., 2015). In European countries such as Italy, Germany, and Slovenia, their use has caused a reduction of colonies of *A.mellifera* bees, leading the European community to ban these in 2013 (Eisenstein, 2015).

Depending on applied concentrations, fipronil may not cause the immediate mortality of bees. They can decrease the toxic effects of the pesticide through the action of enzymes such as glutathione-s-transferase, catalase, and cytochrome P450 (Johnson et al., 2012). In general, the detoxication system converts fat-soluble substances to insoluble substances in an aqueous environment (Berenbaum & Johnson, 2015). The quality and quantity of nutrients can modulate this system, making it extremely important for bees to maintain an adequate diet. Wahl and Ulm (1983) verified increased agrochemical resistance in young bees after receiving a pollen diet of adequate quality and quantity.

Thus, the supply of a protein diet to bee swarms in areas at risk of contamination by agrochemicals can increase the hemolymph protein levels in nursing bees and improve the resistance of bees exposed to the agricultural pesticide fipronil. We tested the hypothesis if protein supplementation would help the honeybee's longevity and locomotion exposed to fipronil pesticide

Materials and Methods

Treatments

The experiment was undertaken at geographic coordinates 22°49′ S; 48°24′W, with a Cfa type climate (subtropical with summer of the higher temperatures) and an average altitude of 623 m.

Six colonies *Apis mellifera* Africanized (Hymenoptera: Apidae) were used, housed in standard Langstroth hives, and distributed randomly into groups, with three colonies each: G1 – Control Group without protein supplementation and G2 – Supplemented Group with 25% crude protein

For the formulation of the diet containing 25% crude protein, we used 47.6% of cornmeal, 47.0% of soybean meal, 5.0% of sugar, and 0.4% of oil, obtained from a single batch FMVZ Feed Factory, UNESP, Botucatu. The calculated nutritional levels were 4,035 kcal of crude energy and 25% of crude protein (Rostagno et al., 2011).

A bromatological analysis was performed to verify the calculated levels of the diet, which obtained values of 26.9% of crude protein and 3,846 of crude energy (lime/g). From the bee bread collected from the experimental colonies, 20% of crude protein and 4,050 crude energy (lime/g) were obtained.

The feed was provided in a paste form (a mixture of ingredients with a standardized amount of honey) and placed on the top bar of the central frame containing the bee swarms, at 100 g per week during December 2017. The leftovers from the rations were removed at the end of each week and weighed to measure consumption. All colonies had free access to nectar and pollen near the apiary.

Harvesting of bees for evaluation protein concentration in hemolymph, longevity, and locomotion of bees

Four weeks after the start of protein supplementation, two frames with sealed brood from the colonies (G1 and G2 groups) were removed, marked, wrapped in tissue (to keep the bees newly emerged in the frame), and kept in an incubator at 32°C and relative humidity of 80% until the emergence of the worker bees (Roat et al., 2014). After the emergence, the newly emerged workers from each beehive were marked in the dorsal side of the thorax using a nontoxic pen (Posca Paint Pens, Mitsubishi Pencils, Japan) and returned to their respective hives. The marked bees were recaptured on their six-day-old, and the concentration of proteins in their hemolymph was measured. So, the honeybees were exposed to fipronil at a concentration of 0.009 ± 0.003 μg/bee ($LD_{50\%}$) (Zaluski et al., 2015), and the longevity and motor activity measured in the laboratory.

Evaluation of the population development of *A. mellifera*

For the evaluation of population development (G1 and G2 groups), two frames containing the queen's oviposition were located in the center of the nest. This nest had its brood area open and sealed. It was evaluated from the first day of the experiment, for four weeks, according to the methodology adapted from Al-Tikrity et al. (1971).

Evaluation of the longevity of bees exposed to fipronil

On the seventh day, the nursing bees from colonies (G1 and G2 groups, totaling 30 bees/treatment) were recaptured, anesthetized in a freezer, and housed in a Petri dish (150 × 20 mm) with perforated lids to ensure ventilation. The bees of G2 received 2μL of a solution containing $LD_{50\%}$ of fipronil in the dorsal side region using a micropipette. The G1 group received 2μL of distilled water.

The Petri dishes were kept in a dark incubator at 32 ± 1°C and relative humidity of 70 ± 10%. The bees received syrup and 50% sugar and water *ad libitum* during all trials. These experiments were performed in triplicate. The number of dead bees was recorded daily, and the dead bees were removed from the plates. The experiment was conducted until all the bees had died.

Evaluation of the motor activity of bees exposed to fipronil

The evaluation of the motor activity of bees was performed according to the methodology described by Zaluski et al. (2015). Motricity was included in the study to evaluate the possible effects on the motor activity of bees after supplementation with a 25% crude protein diet and contamination with agrochemicals. A total of 10 nursing bees were collected from each experimental colony (G1 and G2 groups) and exposed by contact to $LD_{50\%}$ of the agrochemical fipronil.

We used a wooden box in the form of a drawer to perform the locomotion tests, with dimensions of 60 cm long, 40 cm wide, and 4 cm high. The box was capped with a glass plate to observe the bees. A fluorescent lamp was placed at the top of the box to attract the insects via positive phototaxis. The tests were performed in a dark room with the box tilted at 45°. The box presented five lanes with 50 cm for bee observations during the locomotion test. The bees were separated into two groups. One was subject to contact with fipronil at the $LD_{50\%}$, and the other not. Bees belonging to the experimental groups were placed at the box and released simultaneously. The time spent by bees to travel a 50 cm distance was recorded at two different periods: the first was 1h after contamination, and the second was 4h after contamination.

Protein quantification in the hemolymph of bees

A total of 10 nursing bees were recaptured from the colonies (T1 and T2 groups; totaling 30 bees/treatment) and anesthetized on ice for 10 min. Hemolymph was collected using a micropipette through an incision made at the base of the bee's wing (Cremonez et al., 1998). The protein concentration from the hemolymph sample obtained from each hive was measured using the Bradford (1976) method with a Quick Start™ Bradford Protein Assay (Cat. Nº. #5000201; BioRad). The readings were performed on a spectrophotometer (Spectrophotometer Evolution 60 Thermo Fisher Scientific, USA) at a wavelength of 595 nm. Bovine serum albumin was used to prepare the standard curve (Cremonez et al., 1998). The reading of each sample was made in triplicate.

Statistical analysis

The protein concentration in the hemolymph, motor activity, population development, and bee longevity were evaluated using a Student's t-test. In all tests, the results were considered statistically different when $P<0.05$.

Results

During the experimental period, the average consumption of 17.6 ± 13.1% of the crude protein ration provided to the bee swarms was measured. Supplementation with 25% crude protein significantly influenced the offspring in the sealed brood area compared to the control; however, it did not significantly affect the open brood area (Table 1). Protein supplementation did not affect bee survival compared to the control. However, bees of the control group and those supplemented with 25% crude protein and contaminated with fipronil showed 100% mortality 24 h after exposure (Fig 1).

Table 1. Open and sealed brood area (cm^2) of honeybees *Apis mellifera* Africanized supplemented or not with 25% of crude protein.

	Population development	
	Brood area open	**Brood area sealed**
G1	174.4 ± 85.8a	1548.0 ± 531.3a
G2	191.2 ± 67.7a	2335.0 ± 892.0b

Lowercase letters in the column represent a significant difference between the means ($P<0.05$).
G1: without proteic supplementation; G2: supplemented with 25% crude protein.

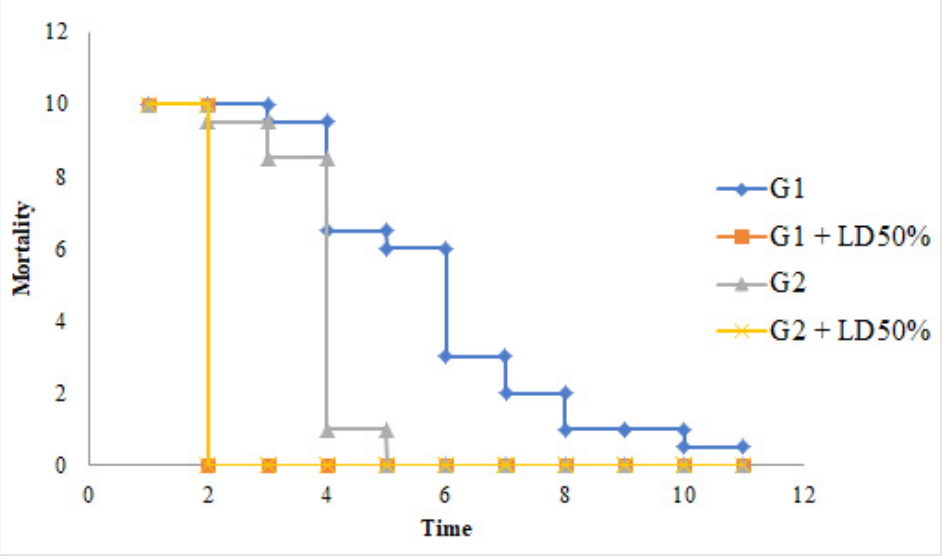

Fig 1. Survival Curve of *Apis mellifera* Africanized supplemented (G2) or not (G1) with 25% of crude protein and challenged or not with $LD_{50\%}$ of fipronil.

In the control group, which did not receive crude protein supplementation, the contamination of bees with $LD_{50\%}$ fipronil affected their locomotion compared to those not contaminated with fipronil for 1 and 4 h. However, there was no difference in locomotion between the bees that received protein supplementation and were contaminated with fipronil compared to bees not supplemented and challenged with $LD_{50\%}$ fipronil for 1 and 4 h (Table 2).

Table 2. Locomotion (seconds) of honeybees *Apis mellifera* Africanized supplemented or not with 25% of crude protein and challenged or not with $LD_{50\%}$ of fipronil.

	Locomotion test	
	1 hour	**4 hour**
G1 control	7.0 ± 3.1a	7.22 ± 2.5a
G1 control + $LD_{50\%}$	14.31 ± 7.6b	18.37 ± 6.9b
G2 + $LD5_{0\%}$	13.13 ± 3.2b	21.13 ± 9.3b

Lowercase letters in the column represent a significant difference between the means (P<0.05).
G1: without proteic supplementation; G2: supplemented with 25% crude protein.

Supplementation did not influence the protein content of the hemolymph of bees compared to the control (Table 3).

Table 3. Protein content in hemolymph of nursing honeybees *Apis mellifera* Africanized supplemented or not with 25% of crude protein.

Hemolymph	
G1	**G2**
18.20 ± 0.36a	18.52 ± 0,60a

Lowercase letters in the column represent a significant difference between the means (P<0.05).
G1: without proteic supplementation; G2: supplemented with 25% crude protein.

Discussion

In the present study, an average intake of 17.6% of the protein diet supplied to the bee swarms was found, corresponding to a 5% intake of the supplied protein. Therefore, the bees supplemented with the protein feed in the field consumed what they needed for their protein needs, which was approximately 25% crude protein (Manning, 2016) because the protein content found in the bee bread harvested from the experimental swarms was 20%.

Although bees can receive their protein needs by foraging floral resources, such as pollen, the protein concentration varies depending on the region and plant variability near the apiary (Forcone et al., 2011). Thus, protein supplementation is important in beekeeping, especially during the off-season period, to help the swarms balance their nutritional needs. Bees can balance their diets to meet their needs (Hendriksma & Shafir, 2016), and protein supplementation showed a positive effect in the sealed breeding area of the swarms compared to the control, suggesting an improvement in the nutrition of bees because the balanced diet could help in the development and reproduction of these insects (Behmer, 2009; Lihoreau et al., 2015).

Protein supplementation of bee swarms did not interfere with the protein content of the hemolymph of the bees, although a direct relationship between these two parameters may occur (De Jong et al., 2009). Nicodemo et al. (2018) verified an increase in hemolymph protein content in bees at seven days of age who had received protein supplementation under laboratory conditions. In the present experiment with protein, supplementation was conducted in field conditions, whith bees performing their normal foraging activities and using the protein provided to balance their needs, which did not promote an increase in the protein content of the hemolymph.

The tests performed under laboratory conditions to evaluate the toxicity of fipronil showed significant mortality of bees with or without supplementation with the protein diet, showing the high toxicity of this agrochemical (Zaluski et al., 2015). Diet supplementation with 25% crude protein did not influence the survival of bees. One way that bees protect themselves from the toxic effects of compounds released in nature is via their detoxification system, which is composed of enzymes such as P450 monooxygenase, glutathione transferase, and carboxylesterase (Li et al., 2007; Rand et al., 2015). Wahl and Ulm (1983) found that young bees showed high resistance after exposure to agrochemicals when they received a protein diet in adequate quality and quantity, suggesting an improvement in the bee detoxification system. However, in the present study, there was no effect of protein supplementation, probably because there was no increase in protein content in the hemolymph of the bee swarms. Thus, the detoxification system was not influenced.

Fipronil significantly affected the locomotor activity of bees, with or without supplementation with 25% crude protein. This result suggests the action of the neurotransmitter gamma-aminobutyric acid (GABA), which is responsible for the reestablishment of the resting state of the central nervous system and muscles of insects (Dowson, 1977; Aajoud et al., 2003; Narahashi et al. 2010). Fipronil may have acted on GABA receptors, promoting neurological and muscular alterations, evidenced in the present study by the locomotor alterations of bees exposed to it. The result of the locomotion test is important because the normal motor activity of bees is essential for their foraging activities, and it was shown that fipronil directly affected this activity, which may compromise the swarm as a whole and promote behavioral, biochemical, and neurological alterations (Sanchez-Bayo & Goka, 2014).

Conclusion

The present study showed that protein supplementation promoted improvement in the development of bee swarms

and is a management practice that beekeepers should adopt, especially during the off-season period when the natural bee forage is reduced. However, this supplementation did not influence the survival of bees when exposed to the agrochemical fipronil, which proved to be toxic for honey bees and promoted changes in their locomotion.

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Authors' Contributions

IRCM: conceptualization, methodology and formal analysis, writing and editing
ROO: conceptualization, methodology and formal analysis, writing and editing
DCBB: writing and editing
JSL: writing and editing

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