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Changes recorded in cardiac graphoelements bees (*Apis mellifera*) during contact with fipronil

Clarissa Araújo da Paz[1] · Luciana Eiró-Quirino[1] · Daniella Bastos de Araújo[1] · Gabriela Brito Barbosa[1] · Tays Mata Câmara[1] · Ana Luiza Cordeiro de Campos[1] · Luana Vasconcelos de Souza[1] · Rafaela Negrão Olívia Santos[1] · Priscille Fidelis Pacheco Hartcopff[1] · Maria Klara Otake Hamoy[1] · Murilo Farias dos Santos[1] · Rayllan da Cunha Ferreira[1] · Yris da Silva Deiga[1] · Antônio Pedro Lisboa Russo[2] · Daniel Santiago Pereira[4] · Felipe Andrés León Contrera[3] · Nilton Akio Muto[2] · Moisés Hamoy[1]

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Abstract
A mortality of bees (*Apis mellifera*) caused by fipronil intoxication, due to its indiscriminate use in crops, has long been attracting the scientific community's attention, either due to its acute or residual effects. In this study, we assessed the cardiac activity as a biomarker of fipronil intoxication through electrophysiological recordings of bees. Eighteen foragers, from the apiary of EMBRAPA—Eastern Amazon (Belém-Pará), bees were previously anesthetized at low temperature (– 10 °C) for 5 min and properly restrained and fixed on a stereotaxic base, where electrodes were implanted. All these procedures were carried out within a Faraday cage. Eighteen bees were used in the study. Worker bees engaged in foraging activities were selected. The bees were divided into a control group and a group treated with fipronil at 0.025 mg/bee (n = 9). The recordings lasted for 4 min and were evaluated at 1-s intervals represented by the following letters: A = (2–3 s), B = (59–60 s), C = (119–120 s), D = (179–180 s), and E = (239–240 s). The results showed that fipronil reduced the frequency and intensity of cardiac activity, exhibiting rapidly evolving effects, and promoting a disruption of homeostasis in bee hemodynamics. Through the obtained data, it was observed variation in spike amplitude, with a loss of cardiac strength and magnitude of the electrical impulse in the bee's heart during exposure to fipronil.

Keywords Pesticide · Heartbeat · Homeostasis · Honey bees · Toxicity

✉ Clarissa Araújo da Paz, clarissa.paz@icb.ufpa.br; Luciana Eiró-Quirino, guimaraeseiro@gmail.com; Daniella Bastos de Araújo, danivivaf@gmail.com; Gabriela Brito Barbosa, gabriela.bribarbosa@gmail.com; Tays Mata Câmara, tays.camara@ics.ufpa.br; Ana Luiza Cordeiro de Campos, ana.cordeiro.campos@ics.ufpa.br; Luana Vasconcelos de Souza, luana.souza@icm.ufpa.br; Rafaela Negrão Olívia Santos, rafaela.santos@ics.ufpa.br; Priscille Fidelis Pacheco Hartcopff, priscille.hartcopff@ntpc.ufpa.br; Maria Klara Otake Hamoy, klara04hamoy@gmail.com; Murilo Farias dos Santos, murilofarias329@gmail.com; Rayllan da Cunha Ferreira, rayllan.ferreira@ics.ufpa.br; Yris da Silva Deiga, yris.deiga@ics.ufpa.br; Antônio Pedro Lisboa Russo, antonio.russo@icb.ufpa.br; Daniel Santiago Pereira, daniel.pereira@embrapa.br; Felipe Andrés León Contrera, felipe@ufpa.br; Nilton Akio Muto, niltonmuto@ufpa.br; Moisés Hamoy, hamoy@ufpa.br | [1]Laboratory of Pharmacology and Toxicology of Natural Products. Institute of Biological Sciences, Federal University of Pará, Belém, Pará 66075-110, Brazil. [2]Center for Valorization of Amazonian Bioactive Compounds, Instituto de Ciências Biológicas, Universidade Federal do Pará, Belém, Pará 66075-110, Brazil. [3]Laboratory of Bee Biology and Ecology, Federal University of Pará, Belém 66075-110, Brazil. [4]Embrapa Eastern Amazon, Belém, Pará 66095-903, Brazil.

1 Introduction

Agriculture's growth has resulted in increased use of insecticides to combat pests in crops, which have been intensively used for over half a century [1, 2]. The application of these products, using various methods, is widespread, particularly in systemic insecticides, with fipronil being one of its representatives [3]. This pesticide is a derivative of the fiprole family [4–6], widely used in cereal and vegetable crops [3, 7].

The effects exerted by fipronil are due to its systemic action related to antagonism in receptors present in chloride channels controlled by gamma-aminobutyric acid (GABA). It competes with GABA for binding sites and, by irreversibly binding to the receptor, prevents nerve impulse transmission, leading to inhibition of synaptic transmission. Additionally, in insects, fipronil can also bind to glutamate-controlled chloride channels, which may explain its differences in selectivity and toxicity in vertebrates and invertebrates [6, 8].

According to Zaluski [9], even at reduced doses, the chemical component in question reveals more severe behavioral alterations in bees through contact than ingestion, mainly due to the lack of enzyme detoxification mechanisms in the digestive system of insects, when contact occurs topically [10]. Holder et al. [11] found a high sensitivity of bees to the toxic effects of fipronil, especially due to its cumulative nature.

The importance of ecological balance, the management of cultivable plant resources, and measures enabling the discovery of appropriate conservation and management techniques that minimally affect insects with zootechnical potential are highlighted [12]. Bees are crucial in ecological systems for its role as pollinators, and as a source of income for beekeepers [13–15]. According to Michener [16], species in the Apini tribe, much like in the Meliponini one, build perennial colonies characterized by morphologically distinct female castes. These colonies reproduce through a process called fission, where the old queen and a group of workers depart to establish a new colony elsewhere. The size of these colonies varies, ranging from a few thousand to over 60,000 workers. However, habitat degradation and a lack of information on bee breeding and sustainable management, along with the effects of pesticides on colonies, are among the main challenges for mapping information as well as effective methodologies for preserving these species [17].

Fipronil is particularly toxic to some species of laboratory mammals upon oral exposure (LD50 = 97 mg/kg for rats; LD50 = 91 mg/kg for mice), but very toxic to bees (LD50 = 0.004 µg/bee) [18, 19]. The Pesticide Action Network [20] has compiled a list of banned pesticides, among which fipronil stands out. This compound has been banned for use and sale in 36 countries, including nations such as Cape Verde and Mauritania, located in Africa, as well as in the United Kingdom, Vietnam and 27 countries belonging to the European Union. A comprehensive analysis of publications related to this pesticide can provide valuable insights for new research projects, the development of detection and degradation methods, as well as guiding environmental policies [21, 22].

Although the literature presents robust data on cardiac function in other experimental models, studies on cardiac function and blood circulation (hemolymph) in bees are relatively scarce and have been carried out especially in the honey bee, *Apis mellifera*, and, to a lesser extent, in the bumble bee, *Bombus terrestris*. Most of these studies are descriptive, but more recent studies have focused on the effects of stressors, mainly pesticides, on the heart activity and other functions [23–25].

In view of the scarcity of articles elucidating these mechanisms, which remain obscure, the aim of this article was to describe the effect of fipronil on the cardiac activity of *Apis mellifera* foragers, evaluating the underlying mechanisms that may alter cardiac recordings.

2 Materials and methods

2.1 Studied species

Eighteen *Apis mellifera* workers were obtained from an apiary from EMBRAPA—Eastern Amazon (Belém-Pará-Brazil) (– 1.4338344019712814, – 48.44947809710463), which were returned to their nest after the experiments. The bees were transported to the Pharmacology and Toxicology of Natural Products Laboratory at the Federal University of Pará—Institute of Biological Sciences, where they were housed in a temperature-regulated environment (26–27 °C) and subjected to the experiments described below.

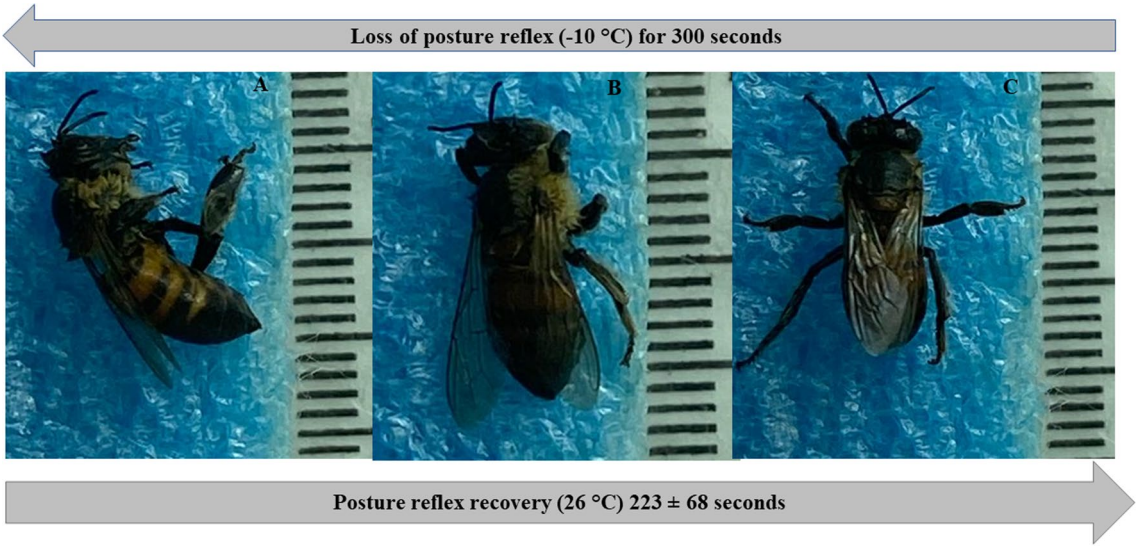

Fig. 1 Characteristics of *Apis mellifera* subjected to (− 10 °C) for 5 min, observing the loss of the laying reflex (indicated by the blue arrow from right to left) (**A**); the animal subjected to (26 °C) begins gradual recovery of reflexes with slow limb movements with a latency of 169 ± 23 s (**B**); followed by recovery of the laying reflex with a latency of 223 ± 68 s (**C**)

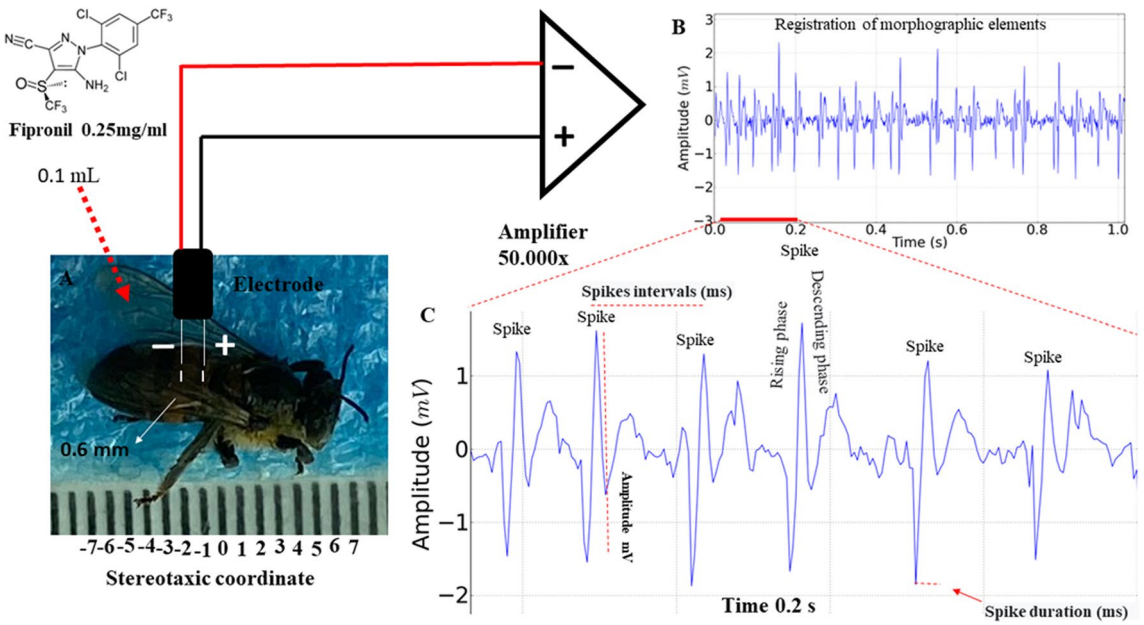

Fig. 2 Stereotaxic coordinates used in animals for signal acquisition, positioning of electrodes spaced 1 mm apart, coupled to a high-impedance amplifier (50,000× signal amplification) (**A**); recording acquisition of recordings with graph elements showing the characteristic signal on a monitor (**B**); amplification of *Apis mellifera* recording, represented by 0.2 s of the trace demonstrating the parameters analyzed allowing the evaluation of Spike Frequency (SPM), Spike Amplitude (mV), Spike Interval (ms) and Spike Duration (ms) (**C**)

2.2 Preparation of bees for experiment

The bees were initially placed in a Petri dish (150 × 20 mm) and subjected to a temperature of − 10 °C for 5 min to reduce their activity and induce the loss of the laying reflex (Fig. 1A). Next, foragers were exposed to a temperature of 26 °C, the laying reflex recovery occurred with an average latency of 223 ± 68 s (Fig. 1B, C). This procedure was necessary to facilitate the fixation of bees on a polyethylene foam platform, with a small elastic band between the thorax and abdomen, and to facilitate electrode implantation (Fig. 2A). Pins were used to secure the bees to the foam and prevent movement. This study followed the method of Contrera et al. [26]. All recordings lasted 4 min, with an initial 2-min accommodation period.

2.3 Electrode fabrication, implantation, and record acquisition

The electrodes were made from JST SM cables with 2-pin Jacks, each 13 cm in length. Nickel–chromium wire electrodes (Morelli Orthodontics), spaced 1 mm apart, with a diameter of 0.2 mm and a length of 2 mm, were insulated with liquid insulation from the brand Quimatic Tapmatic. After the material dried, the electrode was fixed in a stereotaxic device. After bee fixation, the following coordinates were observed, considering the recording electrode as a parameter: the zero point was at the thorax-abdomen intersection on the mid-sagittal line, with an anteroposterior coordinate of 1 mm and a dorsoventral coordinate of 0.6 mm (Fig. 2A). Graph elements were then recorded after the procedure. The entire recording acquisition process was conducted within a Faraday cage. The electrodes were connected to a high-impedance amplifier (Grass Technologies, P511) with a signal amplification of 50,000×, monitored by an oscilloscope (Protek, 6510) (Fig. 2B, C). After being fixed, the bees were exposed to 0.2 mL of a 0.25 mg/mL solution on the animal's back of the insecticide fipronil in spray (30 mL) of the brand formilix from the company formilix Indústria e Comércio located in the municipality of Ibicui, Bahia-Brazil.

All recordings had a duration of 4 min. Fragments were extracted from each recording every minute for analysis and comparison of the effects of Fipronil (0.25 mg/mL) on cardiac activity. Thus, we analyzed the last second of cardiac activity in five different periods: A = 2–3 s (initial period), B = 59–60 s, C = 119–120 s, D = 179–180 s, and E = 239–240 s. Therefore, the experiment consisted of two groups: the control group (n = 9) and the treated group (n = 9) with Fipronil 0.25 mg/mL in a volume of 0.1 mL (0.025 mg/bee), in which the analyzed period was indicated by the letter F (AF, BF, CF, DF, and EF). This period delineation was necessary due to the extensive dataset generated, which would be challenging to visualize if presented continuously. The mean latency for tremors and movement incoordination after fipronil application was 23.14 ± 9.3 s.

2.4 Statistical analyses

The power of record fragments, Spike Frequency (SPM), Amplitude (mV), Spike Interval (ms), and Spike Duration (ms) (Fig. 2B, C) were subjected to tests of normality and homogeneity of variance to assess data variations using the Kolmogorov–Smirnov and Levene tests, respectively. Summarized data are presented as mean ± standard deviation (SD), and p-values are provided. Comparisons between periods were based on two-way ANOVA followed by Tukey's test for multiple comparisons. Statistical analyses and graph constructions were performed using GraphPad Prism, version 8 (Graph-Pad Software Inc., San Diego, CA, USA).

3 Results

In the control group, upon analyzing the records with a duration of 4 min, the average spike frequency was observed to be 1333 ± 91.47 Spikes per minute (SPM), with an average spike amplitude of 1.575 ± 0.3383 mV. The intervals between spikes had an average of 44.56 ± 3.552 ms, and the duration of the spikes was 8.262 ± 1.184 ms. This indicates a sustained rhythm with repetitions of morphographic elements, showing minimal variations (Table 1).

The 4-min records analyzed in *A. mellifera* showed variations in recording amplitude when comparing the control group (Fig. 3A) and the Fipronil-treated group (Fig. 3B). Based on morphographic elements captured by the electrode, larger irregularities can be observed in the records of the Fipronil-treated group (Fig. 3A, B). Fragments were extracted from each record for analysis at the end of each minute of recording and were used to compare power between the groups. During the initial records (2–3 s), the mean powers were similar between the control group ($40.53 \pm 2.289\ mV^2/Hz \times 10^{-3}$) and the fipronil-treated group ($35.41 \pm 3.633\ mV^2/Hz \times 10^{-3}$) (p = 0.0715). At the end of the first minute (59–60 s) after Fipronil application, the power of the cardiac record decreased compared to the control ($24.75 \pm 3.748\ mV^2/Hz \times 10^{-3}$ vs $33.61 \pm 5.125\ mV^2/Hz \times 10^{-3}$). At the end of the second minute (119–120 s), the power of the record decreased further ($24.68 \pm 4.978\ mV^2/Hz \times 10^{-3}$) compared to the control ($33.22 \pm 3.289\ mV^2/Hz \times 10^{-3}$). At the end of the third minute (179–180 s) of Fipronil contact, the recorded power ($9.396 \pm 2.120\ mV^2/Hz \times 10^{-3}$) was lower than the control group average ($27.46 \pm 3.627\ mV^2/Hz \times 10^{-3}$). By the end of the fourth minute of records, the Fipronil-treated group showed an average power of ($8.739 \pm 0.834\ mV^2/Hz \times 10^{-3}$), which was lower than the control group ($27.40 \pm 2.2909\ mV^2/Hz \times 10^{-3}$) (Fig. 3C).

Table 1 Mean of group control analyzed for four minutes of *Apis mellifera* records (n = 9)

Spike frequency (SPM)	Amplitude (mV)	Spike interval (ms)	Spike duration (ms)
1333 ± 91.47	1575 ± 0.3383	44.56 ± 3.552	8.262 ± 1.184

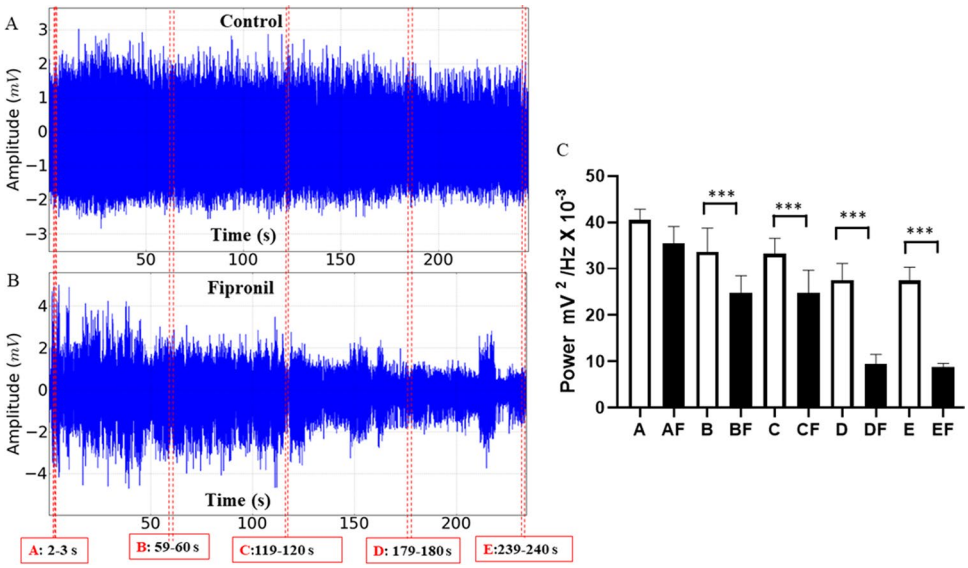

Fig. 3 Tracings represented by 4 min of recording, demonstrating the areas analyzed (dotted in red): A = (2–3 s), B = (59–60 s), C = (119–120 s), D = (179–180 s), and E = (239–240 s) (**A**); tracings recorded in bees in contact with Fipronil 0.25 mg/mL (red dotted): AF = (2–3 s), BF = (59–60 s), CF = (119–120 s), DF = (179–180 s), and EF = (239–240 s) (**B**); the linear power graph of the recorded activity for the control and Fipronil-treated groups in *Apis mellifera* (**C**). (After ANOVA followed by Tukey; *p < 0.001), (n = 9)

For each change in the trace for the control group and the Fipronil-treated group, a pattern of behavior of the recorded graph elements was analyzed over the contact time, always in the final second of each minute: 2–3 s, 59–60 s, 119–120 s, 179–180 s, and 239–240 s. For each record, greater variations in the recorded graph elements can be observed for the Fipronil-treated group (Fig. 4A–E). Therefore, the frequency of spikes in period A was similar between the control (1227 ± 82.48 SPM) and the experimental groups (1303 ± 48.48 SPM) (p = 0.3224). In period B, there was an increase in heart rate for the Fipronil-treated group (1627 ± 85.17 SPM) compared to the control (1379 ± 41.70 SPM). The increased spike frequency for the treated group was maintained in period C with an average of (1754 ± 55.70 SPM) vs. Control (1427 ± 67.83 SPM). In period D, the treated group showed a decrease in spiking frequency (504.4 ± 76.67 SPM) compared to the control (1286 ± 47.53 SPM), and this condition was maintained in period E, with the treated group showing a decrease in spiking frequency (513 ± 81.09 SPM) compared to the control group (1346 ± 55.25 SPM) (Fig. 5A).

The amplitude of the spikes in period A for the Fipronil-treated group (2.503 ± 0.617 mV) was higher than for the control group (1.321 ± 0.159 mV), demonstrating premature alteration of the recorded activity. In period B, the treated group showed a similar mean (2.161 ± 0.492 mV) compared to the control group (1.766 ± 0.256 mV) (p = 0.413), and this relationship was similar for period C, with the mean for the treated group (2.230 ± 0.2324 mV) vs the control group (1.754 ± 0.484 mV) (p = 0.1739). In period D, the control group (1.485 ± 0.277 mV) was higher than the treated group (0.9176 ± 0.213 mV), and in period E, the difference persisted between the control group (1.547 ± 0.257 mV) and the treated group (0.9311 ± 0.143 mV) (Fig. 5B).

For the interval between spikes, there were no significant differences for periods A between the control group (48.11 ± 3.65 ms) and the treated group (45.11 ± 1.45 ms) (p = 0.988). For period B, the control group (42.78 ± 1.39 ms) and the treated group (36.56 ± 2.007 ms) did not show significant differences (p = 0.496). The same relationship occurred in period C, with the mean for the control group (41.11 ± 3.48 ms) vs. the treated group (33.78 ± 1.39 ms) (p = 0.265). However, the interval between spikes increased in the treated group in periods D (115.1 ± 13.49 ms) and E (116.9 ± 12.31 ms) compared to period D of the control (46.44 ± 2.007 ms) and period E (44.33 ± 1.658 ms) (Fig. 5C).

During the recording period, the duration of the spike in period A for the control group (9.48 ± 1.41 ms) was similar to the treated group (9.23 ± 1.19 ms) (p = 0.999). For period B, the control group (8.50 ± 0.65 ms) was greater than the treated group (5.77 ± 0.89 ms). For period C, the control group (8.32 ± 1.01 ms) was greater than the treated group (6.333 ± 1.11 ms), and the same occurred for periods D and E, where the control group (7.55 ± 0.79 ms) and (7.44 ± 0.707 ms) was greater than the treated group (5.11 ± 0.92 ms) and (4.66 ± 0.701 ms) (Fig. 5D).

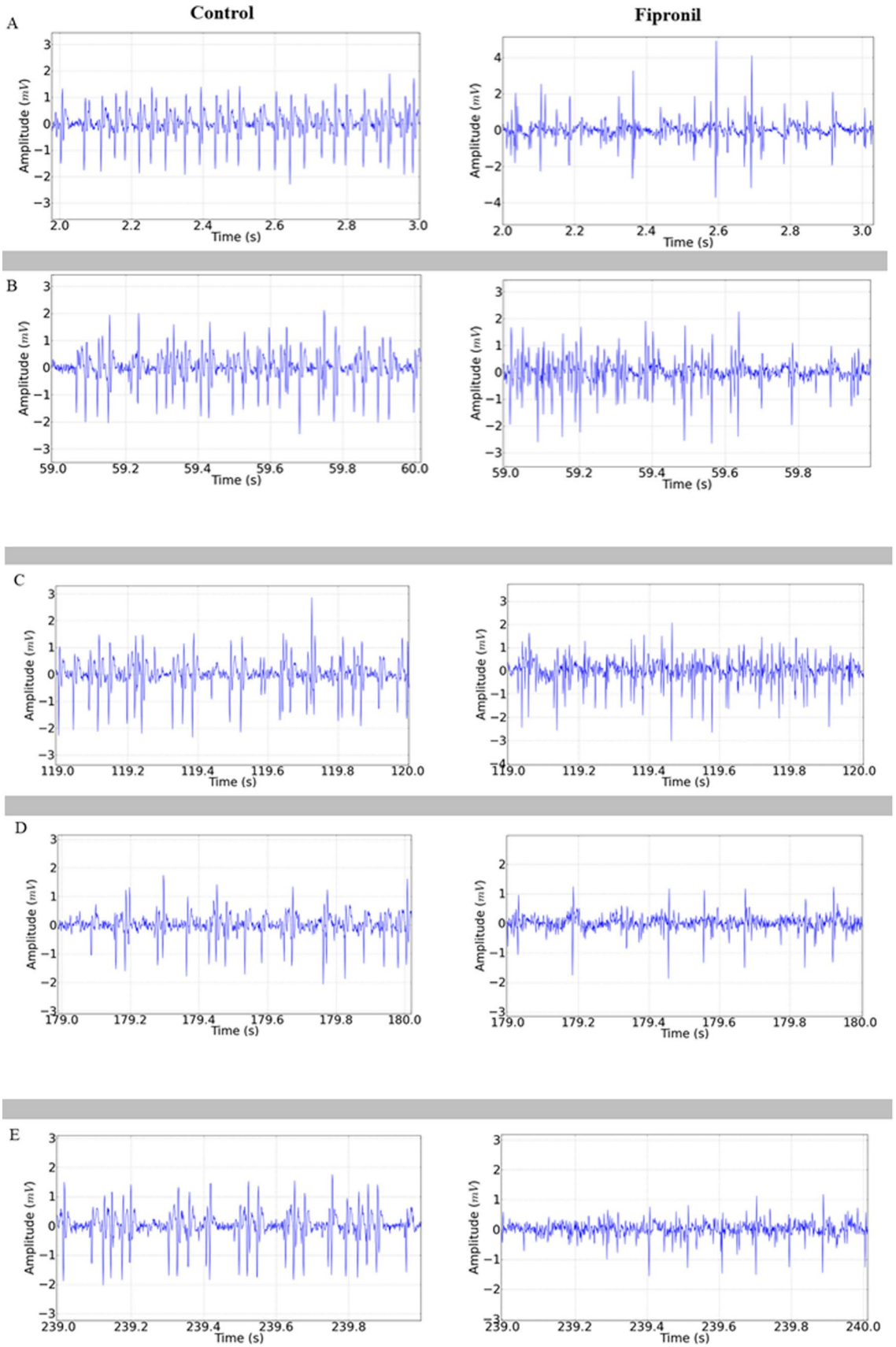

Fig. 4 Patterns of representative tracings for the control group (left) and Fipronil-treated group (right), obtained every minute with a duration of 1 s for the patterns: 2–3 s (**A**); 59–60 s (**B**); 119–120 s (**C**); 179–180 s (**D**); and 239–240 s (**E**)

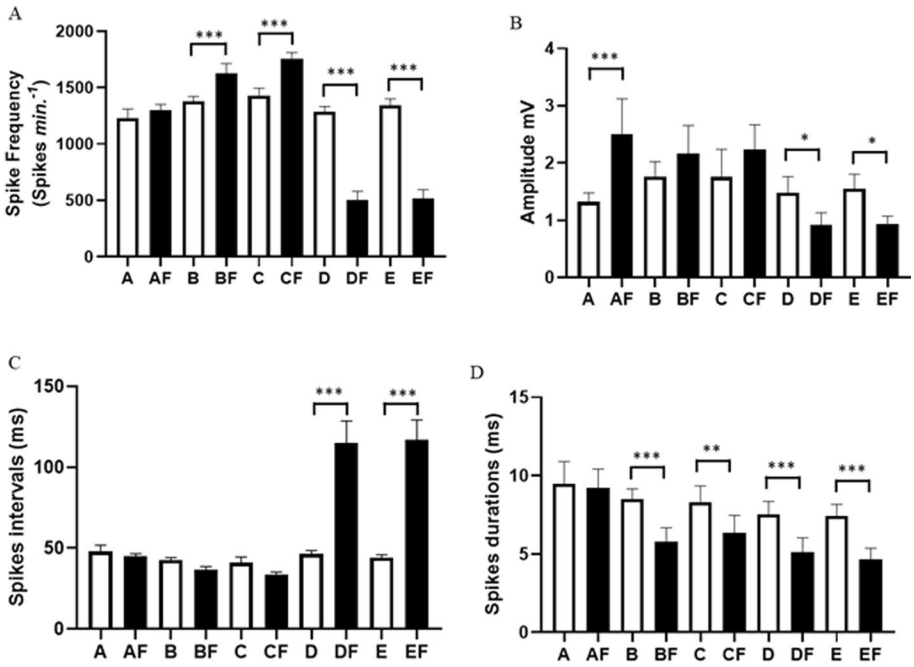

Fig. 5 Evaluation of the recorded activity of *Apis mellifera* for the control and fipronil-treated groups. In each trace, Spike Frequency (SPM), Spike Amplitude (mV), Interval between Spikes (ms), and Spike Duration (ms) were analyzed. After ANOVA followed by Tukey's test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ ($n = 9$)

4 Discussion

Records of *Apis mellifera* cardiac activity have been sparsely reported [27, 28], and the impact of the toxic agent fipronil on assessing the disruption of homeostasis in bee hemodynamics after contact evolves rapidly. Studies demonstrate that the effectiveness of fipronil against the tick, *Rhipicephalus sanguineus*, lasts for 42 days after treatment [29]. This prolonged residual activity should be considered, particularly for more susceptible insect species such as bees.

Fipronil is a widely used pesticide in agriculture for pest control, but, in 2022, the Pesticide Action Network (PAN) pointed out that fipronil was among the pesticides banned in 36 countries. These countries include European Union nations such as the United Kingdom and other regions such as Cape Verde, Mauritania, and Vietnam [22]. These bans were implemented as part of efforts to reduce the risks associated with the use of this pesticide. However, it is associated with the widespread death of pollinating insects [3, 30, 31], as well as systemic effects. It primarily antagonizes GABAA-type chloride channels in invertebrate nerve endings, resulting in a state of excessive stimulation often associated with seizures, ultimately leading to death [32, 33]. After the application of fipronil, the bees exhibited tremors and motor in coordination with a latency of 23.14 ± 9.3 s, indicating rapid absorption and manifestation of effects.

All bee species are susceptible to the neurotoxic mechanisms of fipronil [34]. It is important to emphasize that native stingless bees are also affected by this toxicity, causing functionally detrimental events in various systems, including the endocrine system [35], digestive system [36] and especially the circulatory system. In cardiac activity records, a decrease in signal power occurred over 4 min of recording, intensifying over time with a reduction in power more than three times compared to the control group.

Intoxication by GABAergic neurotransmitter antagonists can induce seizures, and during convulsive episodes, changes in cardiac functionality can occur in various species [37–42]. The records demonstrated an initial momentary increase in the frequency of Spikes followed by a persistent decrease and an increase in irregularities in the graph elements, suggesting arrhythmias. The pattern of heart rate variability caused by fipronil appears to be related to a convulsive crisis associated with damage to the neuronal pathway that regulates cardiac activity [9, 43–47].

In vertebrates, these changes may occur due to the impairment of brain structures that regulate autonomic control, such as the hippocampus, entorhinal cortex, and amygdala [48]. However, in invertebrates, changes found in the hearts of cockroaches of the species *Nauphoeta cinerea*, in a semi-isolated preparation, from exposure to sublethal doses of fipronil also showed a negative chronotropic effect of this compound [49]. Even with extensive washing of the preparations, the heart rate did not recover, suggesting the irreversible nature of these cardiac damages according

to Michener [16]. Changes in cardiac activity, such as a rapid decrease in spike amplitude and frequency, indicate that fipronil has an affinity for bee cardiac cells. The neurological effects caused by fipronil may cause secondary damage to other systems, and by ECG analysis, it could be evaluated the harmful impact of this compound on the species of bees studied. However, further studies are needed to define the mechanism of action linked to fipronil on the cardiac activity of *A. mellifera*. Nevertheless, through electrophysiological tools, it was observed variations in spike amplitude, loss of cardiac force, and magnitude of electrical impulse in the bee's heart during fipronil exposure.

Our results point to the real need for new studies to be carried out so that mechanisms of fipronil action in bees are understood. It includes investigating the neural components involved in regulating the heart rate and directing the cardiac function, as well as exploring the influence of various ion channels, cardiomodulatory peptides and neurotransmitters. It is also necessary to clarify whether the origin of this insect's heartbeat is myogenic, originating within the heart muscle itself, or neurogenic, originating from neural control over the insect's cardiac function and heart rate modulation. By delving deeper into these questions, we can unlock valuable insights into the interaction between physiological processes in bees, thus advancing our understanding of their cardiovascular systems and the maintenance of homeostasis.

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Data availability Data will be made available on request.

Code availability Not applicable.

Declarations

Competing interests The authors declare no competing interests.

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