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FIPRONIL AND IMIDACLOPRID REDUCE HONEYBEE MITOCHONDRIAL ACTIVITY

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Abstract: Bees have a crucial role in pollination; therefore, it is important to determine the causes of their recent decline. Fipronil and imidacloprid are insecticides used worldwide to eliminate or control insect pests. Because they are broad-spectrum insecticides, they can also affect honeybees. Many researchers have studied the lethal and sublethal effects of these and other insecticides on honeybees, and some of these studies have demonstrated a correlation between the insecticides and colony collapse disorder in bees. The authors investigated the effects of fipronil and imidacloprid on the bioenergetic functioning of mitochondria isolated from the heads and thoraces of Africanized honeybees. Fipronil caused dose-dependent inhibition of adenosine 5′-diphosphate–stimulated (state 3) respiration in mitochondria energized by either pyruvate or succinate, albeit with different potentials, in thoracic mitochondria; inhibition was strongest when respiring with complex I substrate. Fipronil affected adenosine 5′-triphosphate (ATP) production in a dose-dependent manner in both tissues and substrates, though with different sensitivities. Imidacloprid also affected state-3 respiration in both the thorax and head, being more potent in head pyruvate-energized mitochondria; it also inhibited ATP production. Fipronil and imidacloprid had no effect on mitochondrial state-4 respiration. The authors concluded that fipronil and imidacloprid are inhibitors of mitochondrial bioenergetics, resulting in depleted ATP. This action can explain the toxicity of these compounds to honeybees. *Environ Toxicol Chem* 2014;9999:1–6.

Keywords: Pesticide Toxicity mechanism Insecticide Honeybee Mitochondria

INTRODUCTION

Approximately 80% of angiosperms require cross-pollination, and more than 30% of these are used by humans in their food. Many insects are also dependent on the plants and use them as a food source and as nesting sites [1]. Impairment of this mutualistic relationship has been observed since the 1940s in the United States, which then had approximately 6 million hives of honeybees (*Apis mellifera*). After 70 yr, hive numbers have been reduced by more than 60%. Annually, mean losses are currently 30% to 40%, resulting in insufficient numbers of colonies for pollination. Many colonies are then divided in the off-season in an attempt to recover colony numbers. This marked population decline also has been observed in other countries and has been termed "colony collapse disorder" [2].

There are several hypotheses that suggest that the disappearance of bees is caused by infestations with mites, nosemosis, or viral diseases. There is also evidence that the population decline of honeybees is caused by the use of pesticides, creating negative consequences for agricultural crops that require bee pollination and for beekeeping [3–6]. Pesticides may also negatively affect bees indirectly by making them more susceptible to parasites and disease organisms [7–9]. In Brazil, the world's largest consumer of pesticides, insecticides accounted for the greatest portion of pesticide sales in 2011, representing 34.7% of the total revenue (US $2.94 billion) and 20.7% of the total consumed commercial product (170 932 metric tons) [10].

The mode of action of pesticides that most frequently affects bees involves the nervous system, with paralysis of the legs, wing muscles, and digestive tract. The insect is not able to feed, and it eventually dies of starvation or desiccation. Consequently, production of honey and various plant products is compromised because of the low frequency of bees visiting flowers. Depending on the level of contamination, the bee affected by the insecticide can return to the hive source and contaminate the food of other bees and the young [11–13].

In both Europe and the United States, European subspecies of bees are used mainly for pollination and honey production. Compared with European bees, Africanized bees (the dominant type found in the tropical and subtropical regions of the Americas) have superior hygienic behavior, are more resistant to pests and diseases, and are more efficient pollinators of cultivated crops; furthermore, they are more dominant and express increased defensive behavior and swarming [14].

Fipronil is a systemic insecticide belonging to the group of pyrazoles, and it acts as a reversible inhibitor of the γ-aminobutyric acid receptor and the inotropic chloride channels activated by glutamate. However, the behavioral effects resulting from sublethal doses are relatively unknown [15]. The median lethal dose (LD50) of fipronil for bees is 13 ng/bee [16]. Imidacloprid is also a systemic insecticide of the group of neonicotinoids, with contact and ingestion action; it acts as a neurotoxin and binds to the nicotinic acetylcholine receptor [17]. The LD50 values of imidacloprid are 3.7 ng/bee and 40.9 ng/bee for oral and contact exposures, respectively [18].

The mitochondrion, an important cell organelle, takes up some substances, such as oxygen and pyruvate, and converts them into energy in the form of adenosine 5′-triphosphate (ATP) for use by the cell. Mitochondria are present in greater numbers in cells of the nervous system, heart, and, in particular in bees, the thorax, because there is a great demand for energy in these parts of their bodies [19]. The pesticides used in modern

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agriculture, in addition to the lethal and sublethal effects already described, may compromise the process of energy production. Energy is indispensable for the maintenance of the colonies; consequently, the demand for bees to seek food in the field is great. In the present study, we investigated the effects of fipronil and imidacloprid on the mitochondrial bioenergetics of the head and thorax of Africanized honeybees to seek an explanation for bee decline.

MATERIALS AND METHODS

Chemicals

Fipronil and imidacloprid were purchased from Sigma-Aldrich. All other reagents were of the highest grade commercially available. Stock solutions of fipronil (1 mM) and imidacloprid (1 mM) were prepared in dimethyl sulfoxide (DMSO) and kept frozen until use. Several dilutions of the stock were made to serve as working solutions. The solutions were protected from light by wrapping the tubes in aluminum foil. The DMSO used to dissolve the compounds had no effect on the assays. Working solutions for isolation and incubation of mitochondria were prepared using glass-distilled, deionized water and were stored at 4 °C.

Animals

Five colonies of Africanized honeybees were used for the present experiment. They were maintained in standard Langstroth hives.

Isolation of thorax and head mitochondria

Mitochondria from the thorax and head were isolated as previously described by Hoskins et al. [20], with modifications. Approximately 20 forager bees were collected from the entrance of each of 5 hives and placed in 1-L clear plastic pots with perforated lids. The same 5 honeybee colonies were used to prepare each pool of bees. Three pools were prepared the same day for each analysis. This was repeated 3 times on different days. The bees were provided with a vial containing 50% sugar syrup and maintained for 24 h in an incubator at 34 °C and 70% humidity. The plastic pots were then transferred to a refrigerator at 4 °C for 15 min to 20 min to cool down and immobilize the bees. The bees were then removed from the refrigerator and decapitated; using scissors and tweezers, the thorax and head were separated into 2 mortars, and the abdomen, legs, and wings were discarded. The mandibles and other mouthparts were removed from the head to increase the percentage of cerebral mitochondria in the final preparation. Thorax (0.75–1.15 g) or head (0.25–0.3 g) tissues were homogenized using a porcelain pestle at 4 °C in 1:20 (w/v) isolation medium (250 mM sucrose, 1 mM ethylene glycol tetraacetic acid [EGTA], and 10 mM *N*-[2-hydroxyethyl]piperazine-*N*′-ethanesulfonic acid [HEPES]–KOH; pH 7.2). The homogenate was filtered through gauze folded 8 times. The suspension was centrifuged at 4 °C for 5 min at 500 *g*, and the resulting supernatant was centrifuged at 4 °C for 10 min at 10 000 *g* to obtain the mitochondrial fraction. The pellet was resuspended in medium containing 250 mM sucrose, 0.3 mM EGTA, and 10 mM HEPES-KOH (pH 7.2) and centrifuged again at 4 °C for 15 min at 10 000 *g*. The final mitochondrial pellet was suspended with medium containing 250 mM sucrose and 10 mM HEPES-KOH (pH 7.2), maintained at 4 °C and used within a maximum period of 3 h. The final concentration of mitochondria in suspension was 20 mg to 30 mg of mitochondrial proteins/mL, determined by a biuret assay with bovine serum albumin as the standard [21].

Mitochondrial respiration assay

Mitochondrial respiration was monitored using a Clark-type oxygen electrode coupled in a sealed glass chamber connected to a 782 Oxygen Meter (Strathkelvin Instruments), and respiratory parameters were determined according to Chance and Williams [22]. One milligram of mitochondrial proteins was added to 1 mL of respiration buffer containing 125 mM sucrose, 65 mM KCl, and 10 mM HEPES-KOH (pH 7.4) plus 0.5 mM EGTA and 10 mM K_2HPO_4, at 30 °C. Oxygen consumption was measured using 4 mM pyruvate (complex I) or 4 mM succinate (+ 2.5 μM rotenone; complex II) as respiratory substrates in the absence (state-4 respiration) or the presence (state-3 respiration) of 400 nmol adenosine 5′-diphosphate (ADP). Oxygen consumption rates were calculated using computer software (Strathkelvin Oxygen 782 System, Ver 3.0). The concentrations of fipronil and imidacloprid (25 μM, 50 μM, 75 μM, and 100 μM) were selected based on a series of pilot studies in our laboratory. Control experiments were performed without addition of the insecticides.

ATP quantification

Levels of ATP were determined using the firefly luciferin–luciferase assay system [23]. After 15 min of incubation in the presence of the same concentrations of substrates and insecticides used in the respiratory test, the mitochondrial suspension (final concentration 1 mg proteins/mL) was centrifuged at 4 °C for 5 min at 9000 *g*, and the pellet was treated with 1 mL of ice-cold 1 M $HClO_4$. After centrifugation at 4 °C for 12 min at 12 000 *g*, 100-μL aliquots of the supernatants were neutralized with 70 μL of 2 M KOH and suspended in 100 mM TRIS-HCl (pH 7.8, 1 mL final volume), and the precipitate was removed by centrifugation at 4 °C for 15 min at 15 000 *g*. Bioluminescence was measured in the supernatant with a Sigma-Aldrich assay kit (catalog FLAA) according to the manufacturer's instructions and measured using a SIRIUS Luminometer (Berthold).

Statistical analysis

The data are expressed as the mean ± standard error of the mean, and significant differences were calculated using one-way analysis of variance followed by Dunnett's test using GraphPad Prism, Ver 4.0 for Windows (GraphPad Software).

RESULTS

Effects of fipronil and imidacloprid on mitochondrial respiration

Mitochondrial oxygen consumption was monitored in the presence of varying concentrations of fipronil (Figures 1 and 2) and imidacloprid (Figures 3 and 4). The parameters assessed were state-3 respiration (consumption of oxygen in the presence of respiratory substrate and ADP) and state-4 respiration (consumption of oxygen after ADP has been exhausted).

At the concentrations tested (25–100 μM), fipronil inhibited the state-3 respiration of mitochondria in a dose-dependent manner. This effect was observed when the mitochondria were energized either with pyruvate (Figure 1), the respiratory chain site-I substrate, or with succinate (Figure 2), a respiratory chain site-II substrate for both the thorax and head. The head mitochondria were more sensitive to inhibition of the respiratory chain (50–100 μM) than the thorax mitochondria (75–100 μM) when the substrate was succinate. Fipronil did not stimulate state-4 respiration, indicating that it does not act as an uncoupler (data not shown).

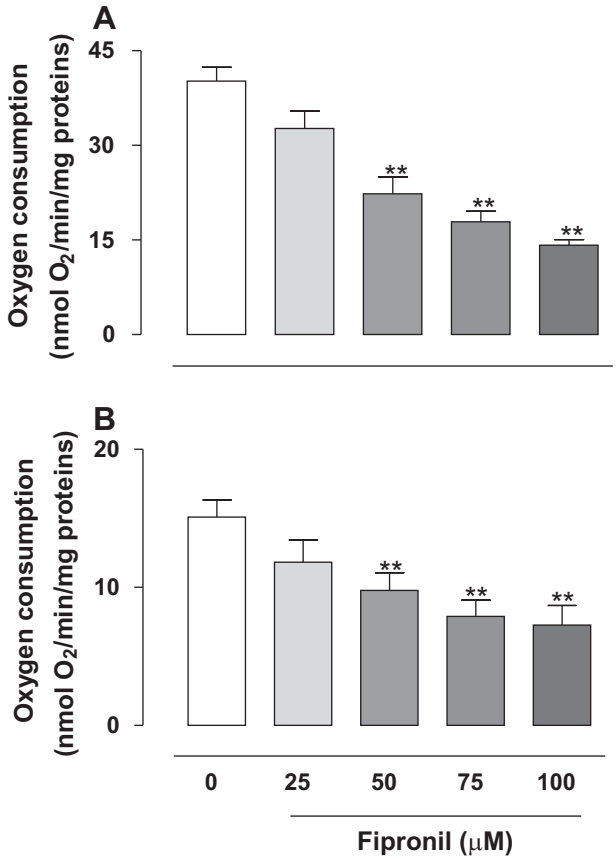

Figure 1. Effect of fipronil on the state-3 respiration rate of pyruvate-energized thorax (**A**) and head (**B**) mitochondria. Values represent the mean ± standard error of the mean of 3 experiments with different mitochondrial preparations. 0 = control, without addition of fipronil. ** Significantly different from control ($p < 0.01$).

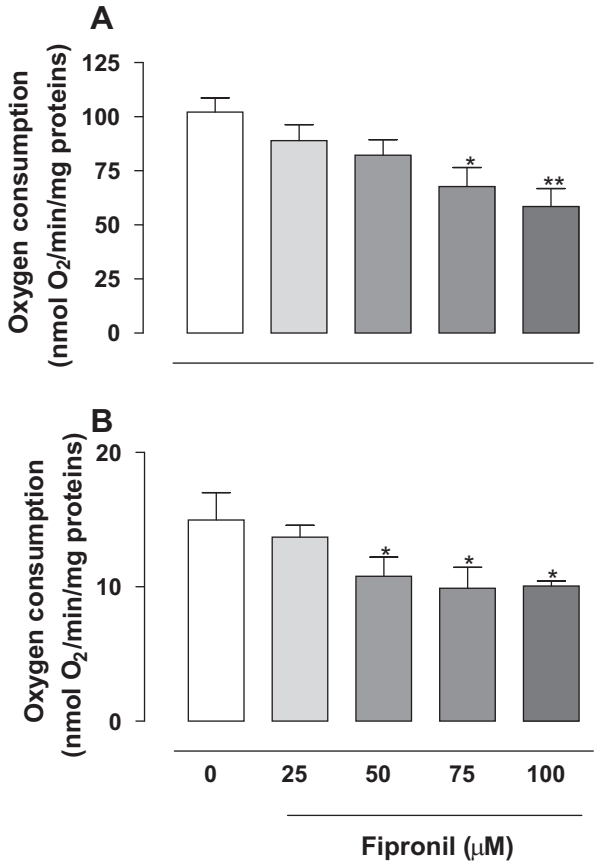

Figure 2. Effect of fipronil on the state-3 respiration rate of succinate-energized thorax (**A**) and head (**B**) mitochondria. Values represent the mean ± standard error of the mean of 3 experiments with different mitochondrial preparations. 0 = control, without the addition of fipronil. Asterisks indicate significant difference from control (* $p < 0.05$; ** $p < 0.01$).

Imidacloprid (25–100 μM) also inhibited the state-3 respiration of mitochondria in a dose-dependent manner for both substrates (Figures 3 and 4). The head mitochondria were more sensitive to inhibition of the respiratory chain than the thorax mitochondria when using both pyruvate (50–100 μM) and succinate (75–100 μM) substrates. Imidacloprid did not stimulate state-4 respiration (data not shown).

Effect of fipronil and imidacloprid on mitochondrial ATP levels

The effect of insecticides on mitochondrial ATP levels in Africanized honeybees was evaluated using respiratory assay conditions after the incubation of mitochondria with the compounds for 15 min (Figures 5 and 6). As observed in the mitochondrial respiration results, fipronil caused a significant dose-dependent decrease of ATP production of the thorax mitochondria with both pyruvate and succinate substrates (25–100 μM; Figure 5A and B), whereas the effect appeared only in the succinate-energized mitochondria in the head; this effect was significant beginning at 50 μM (Figure 5C and D).

Imidacloprid also affected ATP synthesis in a dose-dependent manner, causing significant effects in thoracic mitochondria with both substrates; effects were stronger in mitochondria energized with succinate (75–100 μM; Figure 6A and B). In the head, significant effects appeared only when using the complex II substrate succinate (Figure 6C and D).

DISCUSSION

Although various studies have investigated the effects of insecticides on bees, the bioenergetic performance of the bees has not been evaluated. Mitochondria perform a variety of biochemical processes, but their main function is to produce most (>90%) of the cellular ATP via oxidative phosphorylation [24]. Substrates such as pyruvate and Krebs cycle intermediates present in the mitochondria aqueous matrix space are oxidized by corresponding dehydrogenases, which reduce oxidized nicotinamide adenine dinucleotide (NAD^+) or flavin adenine dinucleotide (FAD). Electron transport from the oxidation of reduced NAD (NADH) and reduced FAD ($FADH_2$) to O_2 is tightly coupled to ATP synthesis. It occurs through protein-bound redox centers, from complex I (NADH-coenzyme Q reductase) or II (succinate-coenzyme Q reductase) to III (coenzyme cytochrome *c* reductase) and then to IV (cytochrome *c* oxidase). The free energy released by this transport is converted by a proton pump into an electrochemical H^+ gradient across the inner mitochondrial membrane. The electrochemical potential of this gradient is then harnessed for the synthesis of ATP by complex V (F_0F_1-ATPase); this process is known as "oxidative phosphorylation." To assess the potential involvement of mitochondria in fipronil- and imidacloprid-related toxicity, we assessed their effects on the bioenergetics of mitochondria isolated from the thorax and head of Africanized honeybees.

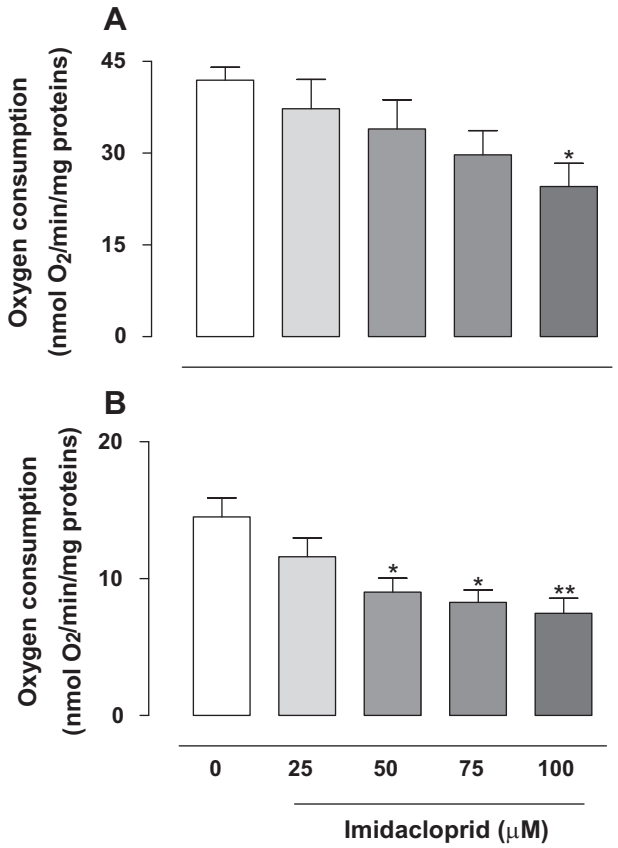

Figure 3. Effect of imidacloprid on the state-3 respiration rate of pyruvate-energized thorax (**A**) and head (**B**) mitochondria. Values represent the mean ± standard error of the mean of 3 experiments with different mitochondrial preparations. 0 = control, without addition of imidacloprid. Asterisks indicate significant difference from control ($^{*}p < 0.05$; $^{**}p < 0.01$).

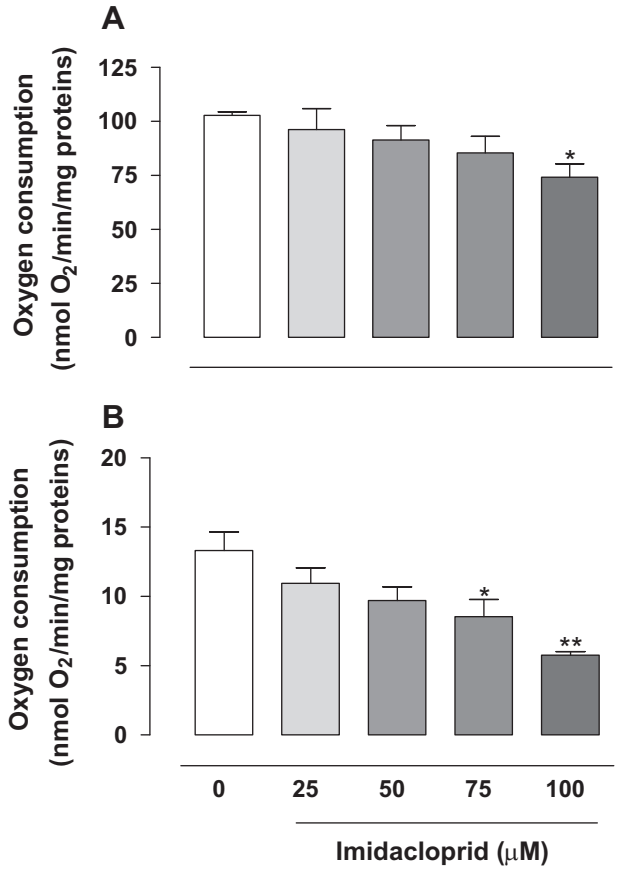

Figure 4. Effect of imidacloprid on the state-3 respiration rate of succinate-energized thorax (**A**) and head (**B**) mitochondria. Values represent the mean ± standard error of the mean of 3 experiments with different mitochondrial preparations. 0 = control, without addition of imidacloprid. Asterisks indicate significant difference from control ($^{*}p < 0.05$; $^{**}p < 0.01$).

The results obtained using mitochondria energized with pyruvate (electron donors to complex I) and succinate (electron donor to complex II) showed that the insecticides inhibit state-3 respiration in a dose-dependent manner at concentrations from 25 μM to 100 μM. According to Chance and Williams [22], , state-3 respiration involves mitochondria, ADP, and a respiratory substrate; and the speed of ADP phosphorylation is the limiting factor of the process. The inhibition observed in the complex I and II experiments indicates that fipronil and imidacloprid have a direct action on the electron transport chain; however, it cannot be ruled out that the insecticides interact with the oxidative phosphorylation system (F_0F_1-ATPase and/or ADP/ATP translocator).

Many xenobiotics cause acute or chronic toxicity through interference with synthesis and use of ATP [25]. In the present study, fipronil and imidacloprid significantly inhibited the synthesis of ATP in a dose-dependent manner, with different sensitivities of thorax and head mitochondria, which could be a consequence of the higher mitochondrial volume densities and cristae surface densities present in the thoracic muscles of flying insects [26].

Though the insecticides more strongly affected oxygen consumption than ATP production, in both cases a tendency to affect mitochondrial activity was observed. These differences could be explained by the fact that inhibition of respiration was not complete; the residual activity of the electron transport chain avoided a significant reduction in the ATP synthesis, as observed in other studies using isolated rat mitochondria [27,28].

In line with our results demonstrating the effect of fipronil on mitochondrial bioenergetics, a study using the Caco-2 cell line, a human intestinal epithelium model, showed that fipronil exposure triggers a perturbation in energetic metabolism, with rapid ATP depletion and glycolysis activation, indicating that fipronil could interact with mitochondria to disrupt oxidative phosphorylation [29]. In addition, fipronil can cause morphological alterations in the larvae of worker honeybee midgut, causing lesions in digestive cells that are characteristic of necrosis, such as cytoplasm vacuolization and electron-dense mitochondria, indicating oxidative stress [30], and promote mitochondrial injury associated with defects of the respiratory process, affecting the neural activity of bees [31].

The toxic effects of insecticides are considered to be involved in the disappearance of bees. Although no single insecticide has been clearly implicated, it is hypothesized that the cumulative and synergistic effects of exposure to various pesticides contribute to the decline in bee populations [5,6,32,33].

According to Sacktor and Wormser-Shavit [34] and Balboni [35], bees cannot fly if their energy metabolism is compromised. Honeybee flight muscles consist of a single fiber type and account for >90% of O_2 consumption during flight [36]. Thus, honeybee energy metabolism is obligatorily aerobic, and most of the ATP synthesis required to drive muscle

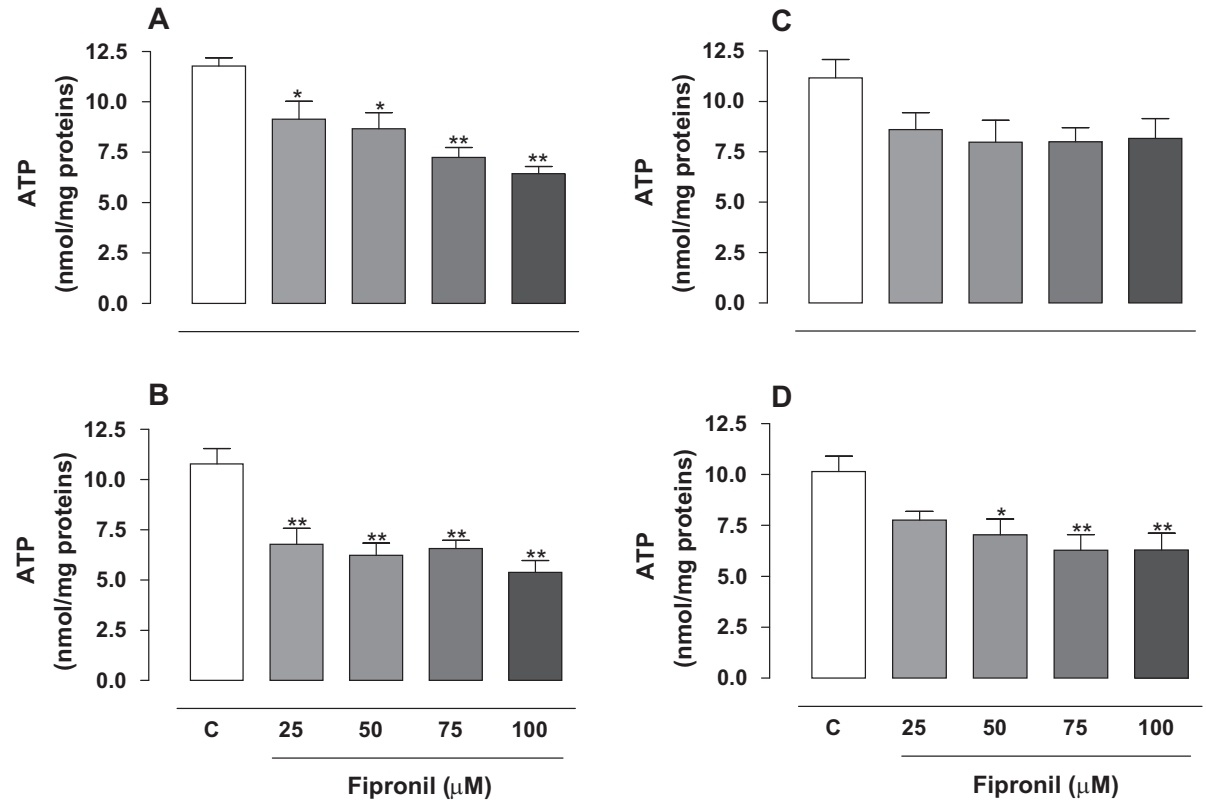

Figure 5. Effect of fipronil on adenosine 5′-triphosphate (ATP) levels in pyruvate- or succinate-energized thorax (**A** and **B**, respectively) and head (**C** and **D**, respectively) mitochondria after 15 min of incubation. Values represent the mean ± standard error of the mean of 3 experiments with different mitochondrial preparations. C = control, without addition of fipronil. Asterisks indicate significant difference from control ($^{*}p < 0.05$; $^{**}p < 0.01$).

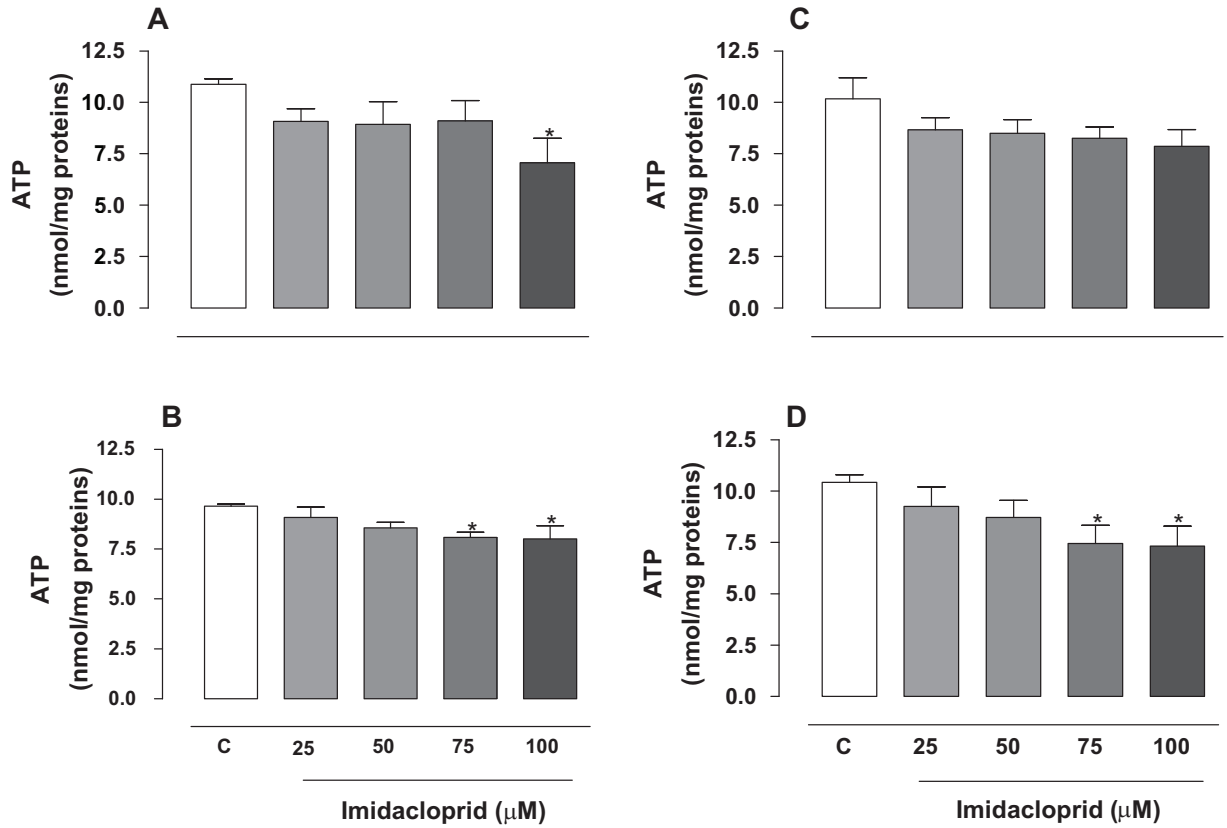

Figure 6. Effect of imidacloprid on adenosine 5′-triphosphate (ATP) levels in pyruvate- or succinate-energized thorax (**A** and **B**, respectively) and head (**C** and **D**, respectively) mitochondria after 15 min of incubation. Values represent the mean ± standard error of the mean of 3 experiments with different mitochondrial preparations. C = control, without addition of imidacloprid. *Significantly different from control ($p < 0.05$).

contraction during flight is derived from mitochondrial oxidative phosphorylation [36–38].

We conclude that fipronil and imidacloprid negatively affect the mitochondrial bioenergetics of the head and thorax of honeybees and that this effect could be involved in the toxicity of these insecticides. Furthermore, our results raise the issue of the risk for human health represented by these insecticides, because field workers may be exposed to high doses during crop treatments.

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