Clomazone herbicide impairs bioenergetics in mitochondria isolated from the thorax of honey bees (Apis mellifera L.)

Herbicida clomazona prejudica a bioenergética em mitocôndrias isoladas do tórax de abelhas melíferas (Apis mellifera L.)

El herbicida clomazona deteriora la bioenergética en mitocondrias aisladas del tórax de abejas melíferas (Apis mellifera L.)

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ABSTRACT
The excessive use of pesticides on crops has caused an increase in animal mortality, contamination of soil, water and food, in addition to being identified as one of the causes of death of bees. Despite being quite effective, the herbicide clomazone (CLZ) causes widespread environmental contamination due to its high solubility in water (1,100 mg.L⁻¹), and its residues can remain in the environment for up to 130 days. Mitochondria are responsible for producing the majority of the ATP in cells, and in bees, the thorax, as it is where the wings are located, is the place with the greatest ATP production. The objective of this work was to evaluate the effects of the herbicide CLZ on mitochondria isolated from the thorax of honey bees (Apis mellifera). The effects of CLZ (100, 125, 150, 200 and 250 µM) on mitochondria were evaluated by determining oxygen consumption, ATP production and generation of reactive oxygen species (ROS). When energized with pyruvate + malate (complex I substrates of the respiratory chain) CLZ did not show significant results in any of the concentrations used, however, when energized with succinate (complex II substrate of the respiratory chain) a dose-dependent stimulus in mitochondrial respiration was observed. Mitochondrial ATP levels were significantly reduced in a dose-dependent manner at concentrations above
125 µM. CLZ did not significantly induce the generation of ROS at any of the concentrations studied. Thus, the results of this study indicate that CLZ toxicity in honey bees may be related to changes in mitochondrial bioenergetics.

**Keywords:** herbicides, pollinizers, bioenergetics, adenosine triphosphate

**RESUMO**
A utilização excessiva de praguicidas nas lavouras tem ocasionado aumento da mortalidade de animais, contaminação dos solos, águas e alimentos, além de ser apontado como um dos causadores de morte das abelhas. Apesar de ser bastante efetivo, o herbicida clomazona (CLZ) provoca ampla contaminação ambiental devido à sua elevada solubilidade em água (> 1 g.L⁻¹), podendo seus resíduos permanecer por até 130 dias no ambiente. As mitocôndrias são responsáveis pela produção da maior parte do ATP nas células, e nas abelhas, o tórax, por ser onde se localizam as asas, é o local com maior produção de ATP. O objetivo deste trabalho foi avaliar os efeitos do herbicida CLZ em mitocôndrias isoladas do tórax de abelhas melíferas (*Apis mellifera*). Os efeitos da CLZ (100, 125, 150, 200 e 250 µM) nas mitocôndrias foram avaliados pela determinação da produção de oxigênio, produção de ATP e geração de espécies reativas de oxigênio (ERO). Quando energizadas com piruvato + malato (substratos do complexo I da cadeia respiratória) a CLZ não apresentou resultados significantes em nenhuma das concentrações utilizadas, porém, quando energizadas com succinato (substrato do complexo II da cadeia respiratória) foi observado um estímulo na respiração mitocondrial de forma dose-dependente. Os níveis mitocondriais de ATP foram reduzidos significativamente de maneira dose-dependente nas concentrações acima de 125 µM. A CLZ não induziu a geração de ERO de maneira significante em nenhuma das concentrações estudadas. Assim, os resultados desse estudo indicam que a toxicidade da CLZ em abelhas melíferas pode estar relacionada às alterações na bioenergética mitocondrial.

**Palavras-chave:** herbicida, polinizadores, bioenergética, adenosina trifosfato

**RESUMEN**
El uso excesivo de plaguicidas en los cultivos ha provocado un aumento de la mortalidad animal, la contaminación del suelo, el agua y los alimentos, además de ser identificado como una de las causas de la muerte de las abejas. A pesar de ser muy eficaz, el herbicida clomazona (CLZ) provoca una amplia contaminación ambiental debido a su alta solubilidad en agua (> 1 g.L⁻¹), y sus residuos pueden permanecer en el medio ambiente hasta 130 días. Las mitocondrias son responsables de producir la mayor parte del ATP en las células, y en las abejas, el tórax es donde se encuentran las alas y es el sitio con mayor producción de ATP. El objetivo de este estudio era evaluar los efectos del herbicida CLZ en mitocondrias aisladas del tórax de abejas melíferas (*Apis mellifera*). Los efectos de CLZ (100, 125, 150, 200 y 250 µM) sobre las mitocondrias se evaluaron determinando el consumo de oxígeno, la producción de ATP y la generación de especies reactivas de oxígeno (ROS). Cuando se energizó con piruvato + malato (sustratos del complejo I de la cadena respiratoria) la CLZ no mostró resultados significativos en ninguna de las concentraciones utilizadas, sin embargo, cuando se energizó con succinato (sustrato del complejo II de la cadena respiratoria) se observó una estimulación dosis-dependiente de la respiración mitocondrial. Los niveles de ATP mitocondrial se redujeron significativamente de forma dosis-dependiente a concentraciones superiores a 125 µM. La CLZ no indujo significativamente la generación de ROS en ninguna de las concentraciones estudiadas. Así pues, los resultados de este estudio indican que la toxicidad de la CLZ en las abejas melíferas puede estar relacionada con alteraciones de la bioenergética mitocondrial.

**Palabras clave:** herbicida, polinizadores, bioenergética, trifosfato de adenosina
1 INTRODUCTION

Pollination is responsible for the production of many agricultural crops around the world. In this process, the pollen grain is transported to the stigma of the flower, either by transferring pollen grains from the anther of one flower to the stigma of another flower of the same or a different species. In return, these plants and flowers produce sweet substances that attract insects (Souza et al., 2007; Imperatriz-Fonseca & Nunes-Silva, 2010). It is estimated that about 75% of the world's food crops depend, at least partially, on insect pollination, with bees being the most effective and important pollinators (Khalifa et al., 2021; Ritchie, 2021). Brazil stands out as a global leader in agricultural production, with many of its crops relying on bee pollination services. Some studies indicate that the economic value of pollination-benefited crops is approximately US$ 15 billion annually (Giannini et al., 2015; Novais et al., 2016; Santos et al., 2018).

In addition to the benefits of pollination, bees are also of great importance in terms of honey production and other beekeeping products. In 2022, the value of honey production in Brazil was about R$ 957 million (IBGE, 2022). The loss of up to 20% of bee colonies is considered acceptable by beekeepers, but since 2006, European beekeepers have experienced up to 80% of their hives being decimated in an unprecedented manner, as worker bees that left in search of food did not return to their original hives, causing the colony to die from starvation and lack of care for the brood. This problem became known as colony collapse disorder (Guimarães, 2007).

Among the probable causes for the disappearance of bees are: colony stress; low genetic variability of queens; use of chemicals in pest control within the colonies; toxins present in the environment; the Varroa destructor mite and associated pathogens; newly discovered pests and pathogens or increased virulence of existing pathogens, and interactions between two or more of these factors (Costa-Maia et al., 2010; Goulson et al., 2015). Although there are no official data on the reduction of the bee population in Brazil, beekeepers and researchers have observed the loss of these insects in recent years, mainly due to deforestation, indiscriminate use of pesticides, and prolonged droughts (Ribeiro, 2017).

Pesticides can lead to the death of bees and the population decline of hives located in areas where these products are applied, causing losses for both beekeepers and farmers. Poisoning can occur through contact, ingestion during visits to flowers, and during occasional fumigation. The action of pesticides most frequently affecting bees occurs in the nervous system, causing paralysis of the legs, wings, and digestive tract. Consequently, the insect does not drink water or feed, dying from starvation or desiccation (Crane & Walker, 1983; Malaspina & Silva-Zacarin, 2006). Many pesticides, even at low concentrations, can be extremely toxic to bees (Thompson, 2001; Van Der Steen, 2001; Thompson, 2002; Rortais et al., 2005). When bees are exposed through contact or food,
these can trigger sublethal effects, such as interference with cognitive abilities, orientation skills, and bee behavior (Pham-Delègue et al., 2002; Rortais et al., 2005).

The herbicide clomazone (2-(2-chlorophenyl)methyl-4,4-dimethyl-3-isoxazolidinone) (CLZ) was developed in the 1980s for the control of annual grasses and broadleaf weeds and is used in a wide variety of crops such as rice, soybeans, corn, cotton, cassava, and sugarcane (Gunasekara et al., 2009; Lippi et al., 2014). Despite being highly effective, CLZ causes extensive environmental contamination due to its high solubility in water (> 1 g.L⁻¹) and a half-life ranging from 28 to 84 days; its residues can persist for up to 130 days in the environment (Colby et al., 1989; Zanella et al., 2002). Hakme et al. (2017) analyzed 22 pollen samples by gas chromatography, collected from hives where bees showed suspicious symptoms of poisoning, and of the samples analyzed, 5% contained CLZ at a concentration of 20 μg.kg⁻¹. Literature data have shown that mitochondria are one of the targets of CLZ-induced toxicity (Fagundes et al., 2015; Cestonaro et al., 2024).

The mitochondria, important cellular organelles, absorbs substances such as oxygen and pyruvate and converts them into energy in the form of adenosine triphosphate (ATP) for use by the cell. Mitochondria are present in greater numbers in the cells of the nervous system, the heart, and, particularly in bees, in the thorax, because there is a high energy demand in this part of the body (Landim, 2009). As demonstrated by Nicodemo et al. (2014) and (2020), in addition to the lethal and sublethal effects caused by pesticide use, these substances can also impair the energy production process in mitochondria, interfering with the activity of worker bees, which are essential for hive maintenance. Thus, the aim of this study was to address the actions of CLZ on mitochondrial bioenergetics by assessing its effects on respiration and ATP levels, in addition to reactive oxygen species (ROS) generation in mitochondria isolated from the thorax of honey bees.

2 MATERIAL AND METHODS

2.1 ANIMALS

An average of 600 bees of the species *Apis mellifera* from standard Langstroth hives were used, originating from the experimental apiary of the College of Agricultural and Technological Sciences of Sao Paulo State University (Unesp), Campus of Dracena. The bees were collected in clear plastic pots containing cotton soaked in water and lids with holes for air entry.

2.2 CHEMICALS

CLZ (Catalog Number 46120; purity 100%) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO). The other reagents used were of the highest commercially available analytical grade. Reagent solutions were prepared with ultra-filtered
water using the Millipore DirectQ-3® purifier system (MilliQ®). The volume of DMSO never exceeded 1% (v/v) of the total medium and had no effect on the assays.

2.3 ISOLATION OF THORAX MITOCHONDRIA

The plastic pots were transferred to a refrigerator at 4°C for 15-20 min to cool down and immobilize the bees. The mitochondria from the thorax of the bees were isolated according to Hoskins et al. (1956), with modifications according to Nicodemo et al. (2014). After separating the head and abdomen, the thorax was added to a porcelain mortar containing 20 mL of medium composed of 250 mM sucrose, 0.2 mM EGTA, 0.1 mM EDTA, 5 mM HEPES-KOH (pH 7.4), at 4°C, and subsequently macerated with a porcelain pestle and filtered through folded gauze 8 times. The suspension was centrifuged at 500 g for 5 minutes at 4°C, and the resulting supernatant was centrifuged at 10,000 g for 10 minutes at 4°C to obtain the mitochondrial fraction. The pellet was suspended in medium containing 250 mM sucrose, 0.3 mM EGTA, and 10 mM HEPES-KOH (pH 7.2) and centrifuged again at 10,000 g for 15 minutes at 4°C. The final mitochondrial pellet was suspended in medium containing 250 mM sucrose and 10 mM HEPES-KOH (pH 7.2) and kept at 4°C. Assays with the mitochondria were performed within a maximum period of three hours.

2.4 PROTEIN ASSAY

The mitochondrial protein was determined according to Cain & Skilleter (1987), using the biuret reaction. An aliquot of 10 μL of the mitochondrial suspension was solubilized in 100 μL of 5% (w/v) deoxycholic acid, followed by 1,390 μL of Milli-Q® water and 1,500 μL of 0.15% (w/v) biuret reagent. The protein concentration was determined at a wavelength of 540 nm using a calibration curve with bovine serum albumin (BSA) as the standard, prepared under the same conditions as the samples.

2.5 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 Limited, Glasgow, Scotland, UK), and respiratory parameters were determined according to Chance and Williams (1956). 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$_2$HPO$_4$, at 30°C. Oxygen consumption was measured using 4 mM pyruvate + 4 mM malate (complex I) or 4 mM succinate (+ 2.5 μM rotenone) (complex II) as respiratory substrates in the absence (state-4 respiration) or the presence of 400 nmol ADP (state-3 respiration). Oxygen
consumption rates were calculated using computer software (Strathkelvin Oxygen 782 System version 3.0). The concentrations of CLZ (100, 125, 150, 200 and 250 µM) were selected based on a series of pilot studies in our laboratory.

2.6 ATP QUANTIFICATION

ATP levels were determined using the firefly luciferin–luciferase assay system (Lemasters & Hackenbrock, 1976). After 10 min of incubation in the presence of 4 mM succinate (+ 2.5 µM rotenone) and the same concentrations of herbicide used in the respiratory test, the mitochondrial suspension (final concentration 1 mg proteins/mL) was centrifuged at 4°C for 5 min at 9,000 g, and the pellet was treated with 1 mL of ice-cold 1 M HClO₄. After centrifugation at 4°C for 10 min at 12,000 g, 100 µL aliquots of the supernatants were neutralized with 70 µL of 2 M KOH, 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 Number FLAA) according to the manufacturer’s instructions and measured using a SIRIUS Luminometer (Berthold, Pforzheim, Germany).

2.7 REACTIVE OXYGEN SPECIES (ROS) PRODUCTION

The production of reactive oxygen species (ROS) by mitochondria from the thorax was evaluated using the probe CM-H2DCFDA (5-(6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate) (Souza et al., 2011). Mitochondria (0.5 mg/mL) energized with 4 mM succinate (+ 2.5 µM rotenone) were incubated with 2 µM CM-H2DCFDA probe and different concentrations of CLZ, and fluorescence was measured in a spectrophotofluorometer model RFPC 5301 (Shimadzu, Tokyo, Japan) at wavelengths of 503 and 528 nm for excitation and emission, respectively. The results were expressed in Relative Fluorescence Units.

2.8 STATISTICAL ANALYSIS

The data are expressed as the mean ± s.e. mean, and significant differences were calculated using one-way analysis of variance (ANOVA) followed by Dunnett’s test using GraphPad Prism, v 4.0 for Windows (GraphPad Software, San Diego, CA, USA). The values of P < 0.05 were considered statistically significant.
3 RESULTS AND DISCUSSION

3.1 EFFECTS OF CLZ ON MITOCHONDRIAL RESPIRATION

The results of oxygen consumption obtained using mitochondria energized with pyruvate + malate, electron donors for complex I of the respiratory chain, did not show significant differences compared to the control (data not shown). However, when mitochondria were energized with succinate, an electron donor for complex II of the respiratory chain, a dose-dependent effect was observed with a significant increase in oxygen consumption from the concentration of 100 µM (Figure 1).

Figure 1. Effect of the herbicide clomazone on the oxygen consumption of mitochondria isolated from the thorax of honey bees energized with 4 mM succinate (+ 2.5 µM rotenone). The results represent the mean ± s.e. mean of 3 experiments with different mitochondrial preparations. *Statistically significant compared to the control (P < 0.05). C = control, without addition of clomazone.

Literature data indicate that CLZ can cause damage to mitochondria in animal cells. Fagundes et al. (2015) reported spontaneous and experimental intoxication by CLZ in sheep and identified mitochondrial swelling with disruption of mitochondrial membranes and lysis of the cristae in neurons, while hepatocytes showed swollen mitochondria with lysed cristae, lipid droplets of varying sizes, and membrane-associated vacuoles with granular content. Cestonaro et al. (2024) evaluated the effect of CLZ on the human monocytic cell line THP-1 and observed that the herbicide causes mitochondrial depolarization with a significant decrease in the mitochondrial membrane potential at concentrations of 1.25, 2, and 4 mM. The results of the present study indicate that clomazone acts as an uncoupler of the respiratory chain and oxidative phosphorylation in mitochondria isolated from the thorax of honey bees. This means that it interferes with the process by which mitochondria produce ATP, the main cellular energy molecule. Normally, the electron transport chain in the mitochondria generates a proton gradient across the inner membrane, which
is used by ATP synthase to produce ATP from ADP and inorganic phosphate. When clomazone acts as an uncoupler, it destabilizes the proton gradient by allowing protons to cross the inner mitochondrial membrane without passing through ATP synthase. This dissipates the membrane potential and reduce the efficiency of oxidative phosphorylation, resulting in lower ATP production (BOELSTERLI, 2007).

3.2 EFFECT OF CLZ ON MITOCHONDRIAL ATP LEVELS

The synthesis of ATP by mitochondria isolated from the thorax of honey bees was inhibited in a dose-dependent manner by CLZ, with significant effects observed from the concentration of 125 µM (Figure 2).

Figure 2. Effect of the herbicide clomazone on the ATP synthesis of mitochondria isolated from the thorax of honey bees energized with 4 mM succinate (+ 2.5 µM rotenone). The results represent the mean ± s.e. mean of 3 experiments with different mitochondrial preparations. *Statistically significant compared to the control (P < 0.05). C = control, without addition of clomazone.

Given that ATP is a fundamental metabolic component, interferences in its synthesis or utilization characterize mechanisms by which xenobiotics can induce acute or chronic toxicity (Meyer & Kulkarni, 2001). There are several reports in the literature of pesticides targeting the mitochondria of bees. For example, Nicodemo et al. (2014) demonstrated that the insecticides fipronil and imidacloprid affect the respiratory chain of mitochondria isolated from the thorax and brain of honey bees (Apis mellifera L.), leading to inhibition of ATP synthesis. The fungicides diniconazole and fludioxonil uncoupled the respiratory chain and oxidative phosphorylation, while dithianon and difenoconazole inhibited the respiratory chain complexes of mitochondria from the flight muscles of bumblebees (Bombus terrestris L.) (Syromyatnikov et al., 2017). Faita et al. (2018), upon exposing honey bees to the herbicide Roundup®, observed the development of morphological and structural alterations in the mitochondria of royal jelly-producing glands that
could diminish their bioenergetic functions and trigger cellular damage related to ATP depletion. Additionally, Nicodemo et al. (2020) demonstrated that the fungicide pyraclostrobin also affects respiration and ATP synthesis in mitochondria isolated from the thorax of honey bees.

In the present study, mitochondria isolated from the thorax of honey bees, which are especially rich in mitochondria due to the high energy demands of flight muscles (Harrison & Roberts, 2000; Syromyatnikov et al., 2019), showed a significant reduction in ATP production in the presence of CLZ. This can lead to an energy deficit that directly affects the bees’ flight capability, reducing their efficiency in pollination and food collection.

3.3 EFFECT OF CLZ ON THE PRODUCTION OF REACTIVE OXYGEN SPECIES (ROS)

Incubation with 100 μM tert-butyl hydroperoxide significantly enhanced the amount of ROS produced by mitochondria isolated from the thorax of honey bees, validating the experiment. No significant changes in ROS production were observed compared to the control at any of the CLZ concentrations used in this study (Figure 3).

![Figure 3. Effect of the herbicide clomazone on the production of reactive oxygen species (ROS) by mitochondria isolated from the thorax of honey bees energized with 4 mM succinate (+ 2.5 µM rotenone). The results represent the mean ± s.e. mean of 3 experiments with different mitochondrial preparations. *Statistically significant compared to the control (P < 0.05). C = control, without the addition of clomazone; C+ = 100 µM tert-butyl hydroperoxide.]

In addition to their crucial role in energy production, mitochondria are also the primary source of reactive oxygen species (ROS) in eukaryotic cells. During cellular respiration, electrons are transferred through the respiratory chain, located in the inner mitochondrial membrane. This process creates an electrochemical gradient that is used by ATP synthase to produce ATP. However, a small fraction of the electrons can escape from the respiratory chain and react with molecular oxygen (O₂), forming superoxide anion (O₂•−). This is the precursor of other ROS, such as hydrogen...
peroxide (H₂O₂) and hydroxyl radical (‘OH) (Murphy, 2009). If not eliminated by the antioxidant system, these species can cause oxidative damage to cellular components like proteins, nucleic acids, and membrane lipids, with serious consequences for the cells (Balieira et al., 2018). The absence of mitochondrial ROS production observed in the present study indicates that oxidation caused by ROS is not involved in the toxicity of the herbicide clomazone in mitochondria isolated from the thorax of honey bees. Our results are in agreement with the previous study by Cestonaro et al. (2024), which also did not demonstrate alteration in the production of ROS in human monocytic cell line THP-1 after 24 hours of incubation with doses of 1.25, 2, and 4 mM of CLZ, i.e., even at doses about 10 to 20 times higher than those used in the present study.

4 CONCLUSIONS

The use of herbicides in agriculture is a common practice for weed control, but their impact on non-target organisms, such as bees, is a growing concern. CLZ, by acting as an uncoupler of the respiratory chain and oxidative phosphorylation and by inhibiting ATP synthesis, causes significant impacts on the bioenergetics of honey bees. The reduction in ATP production can compromise various vital functions of honey bees, affecting their flight capacity, resistance to stressors, and overall hive health. Given the crucial role of honey bees in pollination and agriculture, it is essential to consider the toxic effects of herbicides like clomazone when evaluating their use and regulation.

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